uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Shadia Al-Bahlani

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Cellular and Molecular Medicine)

GRADE / DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Role and Regulation of p73 in Cisplatin-Induced Apoptosis in Human Ovarian Cancer Cells *in vitro*

TITRE DE LA THÈSE / TITLE OF THESIS

Benjamin Tsang

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

Jim Dimitroulakos

Bruce McKay

John Peluso (U. of Connecticut)

Barbara Vanderhyden

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

The Role and Regulation of p73 in Cisplatin-Induced Apoptosis in Human Ovarian Cancer Cells *in vitro*

Mrs. Shadia Mohamed Al Bahlani, MSc.

This thesis is submitted as a partial fulfillment of the Ph.D. program in Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Canada

Your file Votre référence
ISBN: 978-0-494-69129-8
Our file Notre référence
ISBN: 978-0-494-69129-8

ABSTRACT

Chemoresistance is a primary concern in chemotherapyand is an obstacle to successful treatment of human ovarian cancer (OVCA). P73 is important in drug-induced apoptosis in some cancer cells, yet its role in the regulation of chemosensitivity in OVCA is poorly understood. Furthermore, how the deregulation of p73-mediated apoptosis confers resistance to CDDP is unclear. In the present studies, we found that TAp73α over-expression enhanced CDDP-induced PARP cleavage and apoptosis in the chemosensitive OVCA cells OV2008 and A2780s and their resistant counterpart (C13* and A2780cp) in addition to a chemoresistant (Hey cells; the effect of ΔNp73α over-expression was to be found variable. P73α down-regulation attenuated CDDP-induced PUMA and NOXA up-regulation and apoptosis in OV2008 cell. CDDP decreased p73α steady-state protein levels in OV2008, but not in C13*, although the mRNA expression was identical. CDDP-induced TAp73α and ΔNp73α down-regulation in OV2008 was not affected by the presence of proteasome inhibitors (Epoximicin and Lactacystine), nor by the forced expression of Xiap, an anti-apoptotic protein. CDDP-induced, p73α down-regulation was calpain-dependent. CDDP induced calpain activation and enhanced its cytoplasmic interaction and co-localization with p73α in OV2008, but not C13* cells. Calcium (Ca^{2+}) homeostasis plays an important role in apoptosis. We have evaluated the contribution of the Ca^{2+}-mediated, calpain activation and its influence on TAp73α and ΔNp73α contents in CDDP-induced apoptosis. We found that CDDP increased the intracellular calcium concentration ($[Ca^{2+}]_i$) in OV2008 but not C13* while cyclopiazonic acid (CPA), a Ca^{2+}-ATPase inhibitor, caused this response and calpain activation, p73α processing and apoptosis in both cell types. CDDP-induced $[Ca^{2+}]_i$ increase in OV2008

cells was not affected by the elimination of extracellular Ca^{2+}, but stopped by the depletion of internal Ca^{2+} store, indicating that mobilization of intracellular Ca^{2+} stores was potentially involved. These findings demonstrate for the first time that p73α and its regulation by the Ca^{2+}-mediated calpain pathway are involved in CDDP-induced apoptosis in OVCA cells and that disregulation of Ca^{2+}/calpain/p73 signalling may in part be the pathophysiology of CDDP resistance. Understanding the cellular and molecular mechanisms of chemoresistance will direct the development of effective strategies for the treatment of chemoresistant OVCA.

THESIS FORMAT

The current thesis is presented as a classical thesis stated by the guidelines obtained from the Faculty of Graduate and Postdoctoral Studies.

Chapter 1 (Introduction) provides a critical review of the literature on three main areas: a) ovarian cancer and chemoresistance, b) calpain family and c) p73 family and how these areas are related. In addition to what is known on the topics, the current knowledge gaps are also described in this chapter, thus providing the rationale for the work performed in this thesis.

Chapter 2 (Objectives and Hypotheses) presents the overall and specific objectives for conducting the experiments in this thesis. It also states the hypotheses that were tested in the studies.

Chapter 3 (Materials and Methods) consists of a description of the materials that were used during this thesis work, such as cell lines, reagents and antibodies. It also contains a detailed description about the optimizations and experiments performed to test the above hypotheses and achieve the objectives of this thesis.

Chapter 4 (Results) describes the findings of the current thesis. Chapter 4 demonstrates the role of p73α in CDDP-induced apoptosis in ovarian cancer cells and how CDDP regulates its protein content through the calcium/calpain pathway. It also addresses the contribution of the calcium/calpain/p73α pathway to the pathophysiology of CDDP resistance in ovarian cancer cells. These findings resulted in the submission of a manuscript entitled "P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain-dependent mechanism" to ***Oncogene.***

Chapter 5 (Discussion) summarizes and analyzes the findings of the current thesis. It also elucidates the significance and the novelty of these findings and compares them with those previously published. Chapter 5 explains how the findings of this thesis contribute to the field. It also proposes future questions and concerns that should be addressed to fully understand the general concept of the present thesis.

Chapter 6 (References) is a collection of the published papers cited in the present thesis.

Chapter 7 (Appendices) contains additional results that are not fundamental to the major findings of this thesis but are necessary to answer some related questions.

Chapter 8 (Curriculum Vitae) provides an overview of the author's qualifications and the scientific meetings at which the findings were presented.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIF	Apoptosis Inducing Factor
APAF-1	Apoptosis Protease Activating Factor 1
ATM	Ataxia Telangiectasia-Mutated
ATP	Adenosine Triphosphate
ATR	ATM and Rad3-Related
BAX	Bcl-2-Associated X Protein
BCL-2	B-Cell Lymphoma-2
BH	Bcl-2 Homology
Bp	Base Pair
CDDP	Cis-diamminedichloroplatinum (II)
Ca^{2+}	Calcium Ion
$[Ca^{2+}]_c$	Cytosolic Calcium Concentration
$[Ca^{2+}]_i$	Intracellular Calcium Concentration
$[Ca^{2+}]_{ER}$	Endoplasmic Reticulum Calcium Concentration
CDK	Cyclin-Dependent Kinase
CIAP	Calf Intestinal Alkaline Phosphatase
cGMP	Cyclic Guanosine Monophosphate
Chk1	Checkpoint Kinase 1
Chk2	Checkpoint Kinase 2
Cl	Chloride
CPA	Cyclopiazonic Acid
DBD	DNA-Binding Domain

DD	Death Domain
DIABLO	Direct IAP Binding Protein with Low pI
DMEM	Dulbecco's modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DRs	Death Receptors
DSBs	Double Strand Breaks
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylenebis (oxyethylenenitrilo) Tetraacetic Acid
eNOS	Endothelial NOS
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
ERCC1	Excision Repair Cross-Complementing 1
FADD	Fas-Associated Death Domain
FasL	Fas ligand
FIGO	International Federation of Gynecology and Obstetrics
FITC	Fluorescein Isothiocyanate
FLICE	FADD-Like ICE
FLIP	FLICE-Like Inhibitory Protein
FSH	Follicle Stimulating Hormone
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GSH	Glutamylcysteinylglycine
GSTπ	GSH-S-Transferase π

HA	Hemagglutinin
HRP	Horseradish Peroxidase
IAP	Inhibitor of Apoptosis Protein
IgG	Immunoglobulin G
iNOS	Inducible NOS
IR	Ionizing Radiation
JNK	C-Jun N-terminal Kinase
kD	Kilo-Dalton
KCl	Potassium Chloride
LAR	Protein Tyrosine Phosphatase
LH	Luteinizing Hormone
LOH	Loss of Heterozygosity
MAPK	Mitogen-Activated Protein Kinase
MDM2	Murine Double-Minute-2
MDR	Multi-Drug Resistance
MOI	Multiplicity of Infection
Na^+	Sodium Ion
NER	Nucleotide Excision Repair
NFκB	Nuclear Factor Kappa B
NO	Nitric Oxide
NQO1	NAD(P)H Quinine Oxidoreductase 1
OD	Oligomerization Domain
OSE	Ovarian Surface Epithelium

OVCA	Ovarian Cancer
PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly (Adenosine Diphosphate) Ribose Polymerase
PCR	Polymerase Chain Reaction
PH	Pleckstrin Homology
PI3K	Phosphoinositol 3-OH Kinase
PKC	Protein Kinase C
PMSF	Phenylmethylsulfonyl Fluoride
PP1	Protein Phosphatase 1
PTEN	Phosphatase and Tensin Homologue
PUMA	P53-upregulated mediator of apoptosis
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcriptase
sGC	Soluble Guanylyl Cyclase
SDS	Sodium Dodecylsulfate
SERCAs	SR/ER Ca^{2+} ATPases
SEM	Standard Error of the Mean
SMAC	Second Mitochondria-Derived Activator of Caspases
SR	Sarcoplasmic Reticulum
TAD	Trans-Activation Domain
TBS	Tris-Buffered Saline

TBS-T	TBS-Tween
TE	Tris-EDTA
TNF	Tumour Necrosis Factor
TP53	Tumour Suppressive Protein 53
TRADD	TNFR-Associated Death Domain
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRPM	Transient Receptor Potential Ion Channel
UTR	Untranslated Region
UV	Ultraviolet
XIAP	X-linked Inhibitor of Apoptosis Protein

DEDICATION

I dedicate this thesis to:

- ***My father, Mohamed Al Bahlani***

  Who envisioned me being a "doctor" and inspired my determination and motivation through out the PhD journey. He passed away 23 years ago and did not have the chance to see my achievement. I'm sure that if he is still around, he would be proud.

- ***My mother, Alia Al Rashdi***

  Who taught me how to be persistent in accomplishing my goals.

- ***My husband, Mohamed Al Shamsi***

  Who provided me with all the love and support I needed during my PhD journey.

- ***My kids, Al Aiham and Dania***

  Who experienced with me all the joy and happiness of being a mother and a student.

ACKNOWLEDGEMENT

First of all, I would like to deeply thank my supervisor Dr. Benjamin K. Tsang for his continuous and unlimited help, support, guidance and encouragement during the past five years of my PhD studies. Dr. Tsang is not only a great mentor but he also treated all of his laboratory members as his own family. Beside his well-established scientific protocols in the lab, he never ignored the protocols of the social life such as inviting us for lunch and/or dinner for special occasions and arranging for picnics with families. These protocols made us work in a healthy and lovely environment that gives us sufficient courage and energy to solve the obstacles of the laboratory work. Dr. Tsang not only taught me how to do experiments in the lab, but he trained me to be a good thinker. I extended the skill of thinking in my daily life which helps me to be a better wife and a good mother. Dr. Tsang's mentorship provided me with all the skills that I need to be a good scientist that will strive to be the best in any field.

Secondly, I would like to express my great appreciation to the advisory committee members; Dr. William Gibb, Ajoy Basak, and Dr. Jim Dimitroulakos for their helpful comments, precious suggestions and continuous encouragement during my PhD program.

Thirdly, I would like to sincerely thank Sultan Qaboos University, Sultanate of Oman for their financial support through the Graduate Scholarship Doctoral Research Award.

Also I would like to thank the past and present members of Dr. Tsang laboratory for teaching me new experimental procedures and for their excellent suggestions during the progress of my thesis research.

I would like to acknowledge my dearest mother, my brothers and sisters, along with the other family members, for their emotional support and pleasant feelings.

Specifically, I wish to thank Al Motasim Al Bahlani, my brother who never stopped asking me about my PhD and always wanted to know more about my research. I was sure that he does not understand all of what I was saying but I knew that he was so happy for me and his support and encouragement were limitless.

In addition, I would like to thank my friends and the babysitters for their endless and sustained encouragement. From time to time, they were pulling me away from the world of science to the real one to cheer me up and have some fun.

And last but not least, I would like to thank Mohamed Al Shamsi, my husband who believed in me even when I was about to give up. Words can not describe the tremendous help and support that he provided me. Although he is also committed to advance studies, he never complains nor stopped his support. For me, it was impossible to be a PhD student and a mother of two children, but he has made it much easier with his never-ending love and unlimited encouragement and support.

This work could not have been accomplished without any of the preceding people

CHAPTER 1: INTRODUCTION

1.1 HUMAN OVARIAN CANCER

1.1.1 Epidemiology

Ovarian cancer is the most fatal among all the gynecological cancers worldwide. Late diagnoses and drug resistance are the two main factors responsible for the high mortality rate in ovarian cancer patients. In 2009, it is estimated that 2,500 new cases of ovarian cancer will be diagnosed in Canada, with a predictable mortality of 1,750 patients (Canadian Cancer Society, 2009). The corresponding predictions for the United State (US) are 21,550 new diagnoses with 14,600 women dying of ovarian cancer this year (American Cancer Society, 2009).

1.1.2 Risk factors

A number of theories have been proposed as an attempt to explain the etiology of ovarian cancer, two of which are dominant in the field: the "ovulation" and the "gonadotrophin" theories. (Tortolero-Luna and Mitchell 1995).

In the ovulation theory, it is postulated that the rupture of the ovulating follicle damages the ovarian surface epithelium (OSE), thus requiring immediate repair by cell proliferation, therefore creating the potential for abnormal cell proliferation to develop into ovarian cancer. The frequent occurrence of the damage and repair processes over a lifetime increase the chance of errors during replication (Fathalla 1971). The decrease in ovarian cancer risk when the number of lifetime ovulations is reduced, either through pregnancy, lactation and/or the use of oral contraceptives, supports this theory (Whittemore 1993; Modan, Hartge *et al.* 2001).

The gonadotrophin theory states that excessive gonadotrophin exposure enhances estrogenic stimulation of the OSE, potentially leading to malignant transformation of the epithelial cells (Cramer and Welch 1983). Gonadotrophins are hormones secreted by the anterior pituary gland affecting multiple cell types in the testes and the ovaries. The principal gonadotropins related to ovarian function are luteininzing hormone (LH) and follicle-stimulating hormone (FSH). Excessive gonadotrophin would act directly on the OSE, or indirectly, by stimulating estrogen production. Gonadotrophin levels increase with age and remain high during menopause. The age-specific rates of ovarian cancer support the gonadotrophin theory (Beltsos and Odem 1996).

In addition to the above theories, family history is well recognized as one of the major risk factors for ovarian cancer, particularly in familial ovarian cancer (Easton, Matthews et al. 1996). A number of genes have been identified as contributing to this genetic susceptibility. Germ-line mutations in *BRCA1* and *BRCA2* (Breast Cancer 1 and 2) genes increase the risk of developing ovarian cancer by 39 % and 11 %, respectively (Antoniou, Pharoah *et al.* 2003). Other contributing factors include: age, infertility, ovarian dysfunction, irregular menses, and exposure to industrial pollutants (Vo and Carney 2007).

1.1.3 Histological Classifications

Ovarian tumours exhibit diverse histological characteristics and are derived from three types of cells that make up the normal ovary: the surface epithelial cells, germ cells and the sex-cord cells.

The majority of ovarian tumours arise from the surface epithelial cells, which accounts for approximately 90% of ovarian cell tumours (Kaku, Ogawa *et al.* 2003). The epithelial ovarian tumours are further classified according to the predominant pattern of tumour cell differentiation such as: the epithelial cell type, the relative amounts of epithelium and stroma, the presence of papillary processes and the location of the epithelial elements. Serous, mucinous, endometrioid, clear cell, transitional cell, squamous cell and mixed epithelial cell tumours, as well as undifferentiated carcinomas comprise the surface epithelial cell classification.

Germ-cell tumours account for only 5% of ovarian tumours and are considered less aggressive compared with the other types (Canadian Cancer Society, 2009). Usually, germ-cell tumours occur in younger women. Most are benign (teratomas), providing a good prognosis for these young women.

The remaining 5% of ovarian tumours are derived from sex-cord tissues (Canadian Cancer Society, 2009). Such a tumour is formed from cells of the sex-cord, or mesenchyme, which contains gonadotropin-responsive cells (*e.g.,* granulosa cells and thecal cells), as well as fibroblasts. Granulosa cell tumours represent the major subtype of this class, particularly found in women of the 50-55 year age group with post-menopausal, vaginal bleeding due to estrogen secretion by the tumour.

1.1.4 Staging

The International Federation of Gynecology and Obstetrics (FIGO) has established the staging of ovarian cancer based on the post-operative findings of biopsies. Staging is essential for the selection of treatment and as a prognostic factor for patient management. The following are the specific criteia used by the FIGO for ovarian cancer staging (Kaku, Ogawa et al. 2003):

Stage I: Growth limited to the ovary

Stage II: Growth involving one or both ovaries with pelvic extension

Stage III: The presence of tumours involving one or both ovaries with peritoneal implants outside the pelvis or limited to the pelvis with malignant extension to small bowel or omentum

Stage IV: Growth involving one or both ovaries with distant metastasis

Early detection of ovarian cancer significantly increases the survival rate, where 94% of patients live longer than five years after the initial diagnosis (Berek, Bertelsen *et al.* 2000). Unfortunately, the majority of ovarian cancer (80%) is diagnosed at late stages (III or IV) with a five years survival rate of 45% (Holschneider and Berek 2000).

1.1.5 Treatment

The treatment of ovarian cancer is determined by the FIGO staging, typically, by surgery and chemotherapy and rarely by radiation

Surgery is the first line of treatment. Biopsies are obtained by surgical means to determine staging, differential diagnoses and thus, subsequent management. Surgery may be sufficient for the treatment of malignant tumours that are well-differentiated and confined to the ovary (Lehtovirta 2000). Different types of surgery may be performed

depending upon the location and the extent of the tumour; for instance, the removal of either one (unilateral oophorectomy) or both ovaries (bilateral oophorectomy) of a stage III or IV tumour (Trope, Paulsen *et al.* 2000).

Chemotherapy has been a standard treatment for ovarian cancer for decades. Typically, it is used to remove any residual, cancerous tissue that can be missed by surgery. The combination chemotherapy of platinum-based drugs (*i.e.* cisplatin or carboplatin) and paclitaxel is the preferred regimen for ovarian cancer treatment.

Radiation is not a common mode of ovarian cancer treatment; it has not been found to be effective during the advanced stages, when most ovarian cancer patients are diagnosed (Steren, Sevin *et al.* 1993). The high therapeutic dose of radiation used harmfully effects normal tissues of surrounding organs, such as those of the liver and the intestine. When employed for cases of ovarian cancer, radiotherapy may be used to kill cancer cells from a cyst, which has ruptured during the surgical removal of an ovary (Vo and Carney 2007).

1.2 CISPLATIN AND CHEMORESISTANCE

Cis-diaminedichloroplatinum (Cisplatin or CDDP) was first discovered by Barnett Rosenberg in 1965, who found it to be a strong inhibitor of bacterial cell growth (Rosenberg, Vancamp *et al.* 1965). Later, CDDP was developed into a potent platinum-based, anti-cancer drug used widely in the treatment of many solid tumours, including those of the ovary (Cvitkovic and Misset 1996; Gordon, Asmar *et al.* 2004; Gordon, Tonda *et al.* 2004).

Other platinum-based drugs are also used for ovarian cancer, including Carboplatin and Oxaliplatin. Generally, these platinum drugs, as well as CDDP, are known to target DNA. Several studies have suggested that platinum-based drugs may also have extra-nuclear targets, such as the endoplasmic reticulum (ER) and the mitochondria (Mandic, Hansson *et al.* 2003; Gourdier, Crabbe *et al.* 2004).

1.2.1 Cisplatin structure and pharmacokinetics

CDDP is a neutral inorganic compound consisting of two amino groups ($-NH_3$) and two chloride ions (Cl^-) attached to a central platinum ion in the *cis* configuration (Figure 1A) (Jordan and Carmo-Fonseca 2000).

The effects of CDDP are normally influenced by the surrounding chloride ions. Inside the cell, the chloride ion concentration is much lower than in the blood and in the extracellular tissue, thus increasing CDDP reactivity. CDDP easily crosses the cell membrane by losing its chloride ions, forming positively charged platinum (el-Khateeb, Appleton *et al.* 1999). Subsequently, this positively charged complex is attracted to any nucleophilic groups containing oxygen, nitrogen or sulfur atoms with unpaired electrons. These groups are found in many amino acid chains as well as in the purine bases of DNA or RNA; however, the most relevant interactions are those involving DNA forming adducts, including inter- and intra-strand crosslinks (Eastman 1987). The vast majority of these adducts are the 1,2-intra-strand ApG and GpG crosslinks, accounting for 85-90% of the total lesions (Figure 1B) (Kelland 1993; Yang, van Boom *et al.* 1995). These adducts bend and unwind the DNA helix, resulting in cellular DNA damage responses (Zamble and Lippard 1995; Moggs, Szymkowski *et al.* 1997).

1.2.2 Cellular Response to Cisplatin

The cellular response to CDDP is a very complex process. Usually, it starts with detection of the CDDP-induced DNA damage, followed by a series of events, including signal transduction and the activation of transcription factors. The activated transcription factors induces the expression of various genes involved in DNA repair, cell-cycle arrest and apoptosis (Yang, Xu *et al.* 2004). ATM (Ataxia-telangiectasia-mutated) and ATR (ATM and Rad3-related protein) are well known kinases activated at the very early stages of DNA damage response (Durocher and Jackson 2001). They are members of the phosphatidylinositol 3-kinase (PI-3) superfamily, which serve as initiators of the signal transduction process (Lowndes and Murguia 2000; Abraham 2001).

The PI-3 family members are usually large proteins. ATM contains 3056 amino acids, while ATR has 2644 amino acids in its chain. Both of these PI-3s have a C-terminal catalytic domain. Exactly how these two kinases are activated and respond to DNA damage is unknown; typically, they respond to distinct types of DNA damage. ATM is the key mediator of the double-strand breaks (DSBs) response induced by ionizing radiation (IR), unlike ATR, which plays a supporting role in this response. ATR function is directed towards the principle response to UV-induced damage, where the role of ATM is insignificant (Jackson 1997; Wright, Keegan *et al.* 1998; Andegeko, Moyal *et al.* 2001).

ATM and ATR are serine-threonine kinases that phosphorylate cellular substrates involved in regulating cellular response to DNA-damage (Wang 1998; Kim, Lim *et al.* 1999). It has been shown that ATM and ATR phosphorylate checkpoint kinases, Chk1 and Chk2 (Matsuoka, Huang *et al.* 1998; Zhao and Piwnica-Worms 2001; Gatei, Sloper *et al.* 2003; Goudelock, Jiang *et al.* 2003; Ng, Lee *et al.* 2004; Wang, Wiltshire *et al.* 2004). The initiation of DNA repair and the regulation of cell-cycle arrest and/or apoptosis are related to these checkpoint kinases. The ATR/ATM kinases also regulate p53 activity at multiple levels by directly phosphorylating p53 at various sites (serine 6, 15 and 46) (Banin, Moyal *et al.* 1998; Canman, Lim *et al.* 1998). Indirectly, they also activate the up-stream regulators of p53 such as c-Abl (Non-receptor tyrosine kinase) and Chk2, which in-turn induces p53 phosphorylation (serine 20) (Chehab, Malikzay *et al.* 1999; Hirao, Kong *et al.* 2000). The ATM/ATR-induced modifications of the p53 protein enhance its transcriptional activity and stabilization, and thus its function.

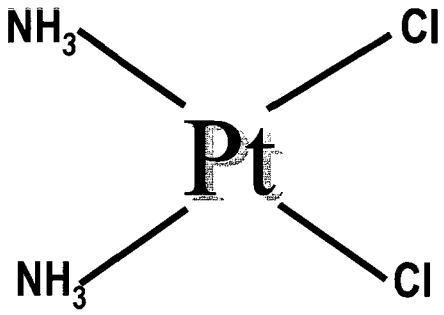
Figure 1.1: CDDP structure and its DNA-adducts

Adapted from (Torigoe, Izumi et al. *2005).*

A) CDDP is a neutral inorganic compound consisting of two amino groups ($-NH_3$) and two chloride ions (Cl^-) attached to a central platinum ion in the *cis*-configuration.

B) Intrastrand 1,2- (ApG and GpG) are the major CDDP crosslinks (85-90% of total lesions). The minor crosslink is the 1,3-GpNpsG. CDDP also causes the interstrand crosslink (i.e. 1,2-GG), which is less common than the above crosslinks.

A


NH3
Cl
Pt
NH3
Cl

B

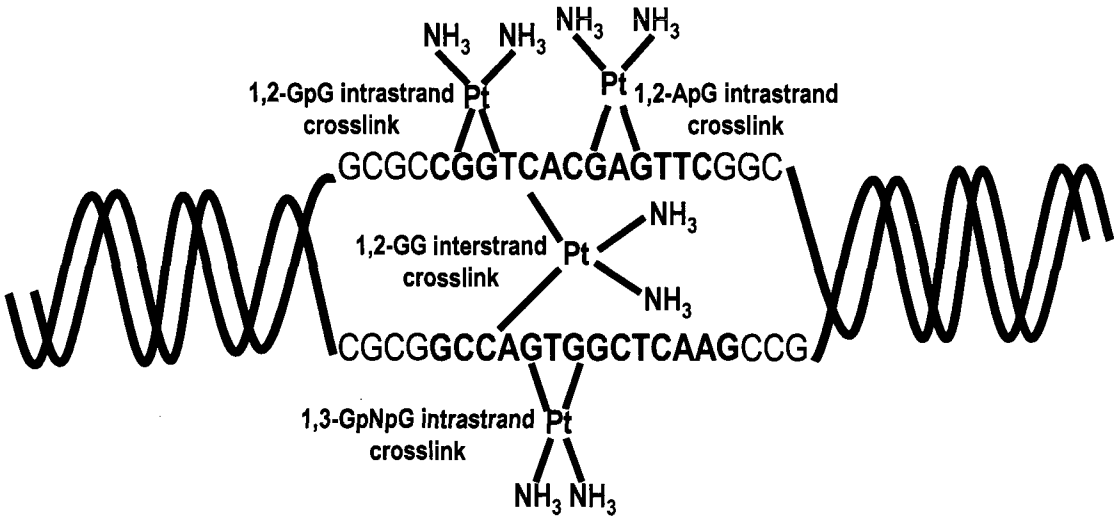

NH3 NH3
NH3 NH3
1,2-GpG intrastrand crosslink
Pt
Pt
1,2-ApG intrastrand crosslink
GCGCCGGTCACGAGTTCGGC
1,2-GG interstrand crosslink
Pt
NH3
NH3
CGCGGCCAGTGGCTCAAGCCG
1,3-GpNpG intrastrand crosslink
Pt
NH3 NH3

Figure 1.1

1.2.3 Mechanisms of resistance

Although CDDP is an effective anti-cancer drug for the treatment of ovarian cancer, its efficacy is often limited by the development of resistance. The mechanisms underlying CDDP-resistance have been widely studied and they can be categorized into four key alterations as briefly discussed below. These mechanisms are: reduced intracellular drug accumulation, increased inactivation by thiol-containing molecules, increased DNA repair and inhibition of apoptosis.

Inhibition and/or enhancement of drug uptake may be attributed to CDDP accumulation. Recent evidence has suggested a link between copper transporter and CDDP resistance. It had been thought that CDDP enters the cell by passive diffusion; however, current reports show that transporters controlling copper influx, especially Ctr1, are involved in the cellular uptake of CDDP (Lin, Okuda *et al.* 2002; Kuo, Chen *et al.* 2007). Ctr1 is a highly conserved membrane-spanning P-type ATPase; it is specialized for copper and the knock-out of this protein in murine embryonic fibroblasts has led to the reduced uptake of CDDP (Holzer, Manorek *et al.* 2006).

On the other hand, the over-expression of the transporters controlling copper efflux, (*e.g.* ATP7 and ATP8) cause CDDP resistance (Komatsu, Sumizawa *et al.* 2000). High levels of ATP7 mRNA in ovarian cancer have been correlated with the decrease in sensitivity to CDDP (Kalayda, Wagner *et al.* 2008; Blair, Larson *et al.* 2009). The increased expression of ATP7 transporters in patients with ovarian cancer was associated with poor survival rates in patients (Samimi, Varki *et al.* 2003; Nakayama, Kanzaki *et al.* 2004).

Like the influence of the copper transporter in CDDP accumulation, the multidrug resistance (MDR) P-glycoprotein (P-gp) pump has been associated with the control of CDDP efflux and thus, CDDP resistance. A clinical study with CDDP-based treatment in advanced ovarian cancer has demonstrated that the over-expression of such protein is associated with a poor chemotherapeutic outcome (Baekelandt, Holm *et al.* 2000). In addition, the MDR/P-gp expression was found to be higher in patients who were not responding to CDDP treatment, compared with those who did respond (Kamazawa, Kigawa *et al.* 2002). Taken together, these results suggest that MDR/P-gp may be an important determinant of the CDDP-resistance in ovarian cancer.

Many exogenous toxins, including CDDP, can be detoxified by glutathione (γ-glutamylcysteinylglycine, GSH) through the formation of GSH adducts. The elevated reactivity of the hydrolyzed CDDP in the cell promotes its interaction with GSH, either in a non-enzymatic manner, or via the GSH-S-transferase π (GSTπ) enzyme (Siddik 2003). The expression of GSH, together with the activity of GSTπ, increases in resistant ovarian cancer cells compared with their parental cells (Oguchi, Kikkawa *et al.* 1994; Goto, Yoshida *et al.* 1995). Additionally, the GSH concentration has been found to be elevated in the primary cultured ovarian cancer cells taken from the same patients after CDDP treatment (Wolf, Hayward *et al.* 1987). The acquisition of CDDP resistance in ovarian cancer cells may, in part, be due to the high rate of CDDP deactivation and exclusion caused by the relative GSH level, and the subsequent GST reaction.

CDDP adducts are mainly repaired by the nucleotide excision repair (NER) pathway (Lindahl, Karran *et al.* 1997). The NER pathway recognizes and removes bulky lesions in the DNA double helix. Once these lesions are removed, a single-strand gap in

the DNA remains. Subsequently, DNA polymerase ligates the remaining single-stranded fragment.

Enhanced DNA repair is another factor that may confer CDDP resistance; cells deficient in NER are hypersensitive to CDDP (Plooy, van Dijk *et al.* 1985; Dijt, Fichtinger-Schepman *et al.* 1988). Of the various proteins involved in the NER pathway, the up-regulation of only a few rate-limiting proteins are enough to increase the NER capacity in resistant tumour cells (Reed 1998). Several studies have demonstrated a clinical correlation between CDDP resistance and the increased level of the excision repair cross-complementing 1 (ERCC1) protein in ovarian cancer (Dabholkar, Vionnet *et al.* 1994; Li, Yu *et al.* 2000). The down-regulation of this protein has been found to sensitize ovarian cancer cells to CDDP (Selvakumaran, Pisarcik *et al.* 2003).

While chemoresistance may be attributed to altered DNA repair, drug transport and metabolism, CDDP resistance in ovarian cancer can also be associated with impaired, drug-induced apoptosis. This results from the up-regulation and reduction of either the pro- or anti-apoptotic factors (Eltabbakh and Awtrey 2001).

Sensitivity to apoptosis is known to be essential for the therapeutic responses of cancer cells to CDDP treatment. Apoptotic pathways are triggered by CDDP and any alteration in their regulation may partially confer resistance. Such alterations are usually induced by apoptotic inhibitors, including the X-linked inhibitor of apoptosis protein (Xiap) and survivin (a member of the inhibitor of apoptosis protein (IAP) family). Xiap is a determinant of CDDP-resistance, where its over-expression has attenuated CDDP-induced apoptosis in ovarian cancer cells (Asselin, Mills *et al.* 2001; Fraser, Leung *et al.* 2003; Mansouri, Zhang *et al.* 2003). The progression of ovarian tumours and CDDP

resistance in ovarian cancer cell lines has been linked to the high expression of Survivin (Zhang, Pan *et al.* 2006). Enhancing CDDP-induced apoptosis by modulating its activators and/or inhibitors could be an essential tool in overcoming CDDP-resistance *in vitro*.

1.3 PROGRAMMED CELL DEATH (APOPTOSIS)

Apoptosis, also known as programmed cell death (PCD), can occur under both physiological and pathological conditions. The term programmed cell death, introduced in 1964, indicates that cell death during development does not happen randomly, but follows a sequence of controlled steps leading to defined self-destruction (Lockshin and Williams 1965). The term "apoptosis" means "falling off" in Greek, in an analogy to leaves falling off of trees (Kerr, Wyllie *et al.* 1972).

Apoptotic cell death is an active and defined process. It plays a vital role in the development of multicellular organisms and in the regulation and maintenance of cell population. Apoptosis engages a series of biochemical events that leads to changes in cell morphology. These changes are characterized by membrane blebbing, the loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Saraste and Pulkki 2000).

The apoptotic cellular changes reflect the selective proteolytic degradation of intracellular polypeptides that are cleaved by intracellular cysteine proteases called caspases (Porter, Ng *et al.* 1997; Earnshaw, Martins *et al.* 1999). Eleven caspases have been identified in humans. They are classified into two types: initiator (*i.e.,* caspase 2, 8, 9 and 10) and effector (*e.g.,* caspase 3, 6 and 7) caspases. The initiator caspases cleave the inactive form of effector pro-caspases, thereby activating the latter. To trigger

apoptosis, the activated effector caspases cleave other cellular substrates including: Inhibitor of caspase-activated DNase (ICAD) (Liu, Zou *et al.* 1997; Enari, Sakahira *et al.* 1998) and poly(ADP)ribose polymerase (PARP) (Tewari, Quan *et al.* 1995). Both types are involved in two distinct apoptotic pathways: the death receptor (extrinsic) and the mitochondrial (intrinsic) pathways.

1.3.1 Cell-Death Receptor Pathway

The death-receptor pathway (Figure 1.2) is initiated by the death receptors (DRs). Found on cell surfaces, DRs transmit apoptotic signals following their binding to specific ligands. These receptors belong to the superfamily of TNFR (Tumour Necrosis Factor Receptor). The best-characterized TNFR and ligands are Fas/FasL (CD95/Apo1), TNFα/TNFR1 (Tumour Necrosis Factor Receptor-1) and TNF-related apoptosis-inducing ligand receptor 1 and 2 (Tartaglia, Rothe *et al.* 1993; Dhein, Walczak *et al.* 1995; Ahmed, Bar-Peled *et al.* 1997).

The DRs are characterized by a Cysteine-rich, extracellular domain and a homologous, intracellular domain known as the death domain (DD). Upon ligand binding, the DD interacts with an adaptor protein, the Fas-associated death domain (FADD), either directly or indirectly via the TNFR-associated death domain (TRADD) (Thomas, Henson *et al.* 2004). The FADD binds to pro-caspase-8 forming a complex at the receptor: the death inducing signaling complex (DISC). Once assembled, a DISC activates pro-caspase-8, precipitating the activation of downstream effecter caspases.

In addition to the DRs, the TNF superfamily is comprised of decoy receptors (DcR), which act as antagonists to the DRs, thereby inhibiting their death signaling through ligand competition. For instance, DcR1, DcR2 and osteoprotegerin (OPG) bind to TRAIL and DcR3, which together bind to the Fas ligand (Gurney, Marsters *et al.* 1999). The death receptor signaling is also regulated by cellular FADD-like interleukin 1 β-converting enzyme (FLICE)-like inhibitory proteins (FLIP), which is an endogenous inhibitor that interacts with FADD in order to antagonize apoptosis (Irmler, Thome *et al.* 1997; Abedini, Qiu *et al.* 2004; Abedini, Muller *et al.* 2009)

1.3.2 Mitochondrial Pathway

The mitochondrial pathway (Figure 1.2) occurs in response to stresses, including DNA damage, chemotherapeutic agents, serum starvation and ultra-violet (UV) irradiation. It begins with the permeabilization, then the rupture of the mitochondrial outer membrane, which causes the release of certain apoptotic proteins (Crompton, Virji *et al.* 1999). The most effective of the released proteins include: cytochrome c (Yang and Cortopassi 1998), apoptosis inducing factor (AIF) (Lorenzo, Susin *et al.* 1999), endonuclease G (Li, Luo *et al.* 2001), second mitochondria-derived activator of caspases/direct IAP-associated binding protein with low pI (Smac/DIABLO) (Du, Fang *et al.* 2000) and high temperature requirement A2 (Omi/HtrA2) (Suzuki, Imai *et al.* 2001).

Once released into the cytosol, cytochrome c binds to an adopter protein known as apoptosis protease activating factor (APAF-1). Together, they recruit pro-caspase 9, forming a complex called an 'apoptosome' (Zou, Li et al. 1999). Eventually, this complex activates caspase 9, leading to the activation of effector caspases, which initiate apoptosis. Both AIF and endonuclease G contribute to DNA fragmentation and subsequent chromosomal condensation. Smac and Omi antagonize the IAPs, thereby promoting caspase activation (Du, Fang *et al.* 2000; Suzuki, Imai *et al.* 2001).

Mitochondrial membrane permeability is regulated by the Bcl-2 family members, featured by Bcl-2 homology (BH) domains (Droin and Green 2004). Proteins of the Bcl-2 family are characterized into anti- (*i.e.* Bcl-2 and Bcl-xL) and pro-apoptotic (*i.e.* Bcl-2-associated X protein (Bax), p53-up-regulated mediator of apoptosis (PUMA) and NOXA) members based upon their ability to either suppress or to induce the release of the mitochondrial apoptotic proteins, respectively (Wei, Lindsten *et al.* 2000; Shi 2001; Letai, Bassik *et al.* 2002). Recent studies indicate that p53, in addition to action at the nucleus, also targets the mitochondria to release death proteins in ovarian cancer cells following CDDP treatment (Yang, Fraser *et al.* 2006).

Figure 1.2: The extrinsic and intrinsic apoptotic pathways.

Adapted from (Kaufmann and Earnshaw 2000)

The extrinsic pathway is initiated through the activation of cell-death receptors (*i.e.* Fas) by their specific extracellular ligands (*e.g.* FasL), which leads to the recruitment of adapter molecules such as fas-associated death domain (FADD) and the activation of pro-caspase-8 to caspase-8.

The intrinsic pathway is activated by cell stress, such as DNA-damage. ATM (Ataxia-telangiectasia-mutated) and ATR (ATM and Rad3-related protein) are activated at the very early stages of DNA damage response. Once activated, ATR and ATM kinases induce p53 and/or TAp73 activation through post-translational modifications (*e.g.* phosphorylation). P53 and/or TAp73 induce transcriptional activation of p53-responsive gene products, which in turn mediate the apoptotic pathways.

The intrinsic pathway begins with the activation of the BH_3-only pro-apoptotic Bcl-2 family members, which attenuate the inhibitory binding of the anti-apoptotic family members to Bax. Bax then facilitates mitochondrial membrane permeability and subsequently, the rupture of the mitochondrial outer membrane. This results in the release of apoptotic proteins, including cytochrome c, AIF, Smac and Omi. Once released, cytochrome c binds to an adopter protein, apoptosis protease activating factor (APAF-1), together recruiting and activating pro-caspase 9. Both Smac and Omi antagonize the action of IAPs, thereby promoting caspase activation. AIF contributes to DNA fragmentation and chromosomal condensation.

Eventually, the activated caspase-8 and -9 induce caspase-3 activation. In turn, cellular substrates are cleaved, including Inhibitor of caspase-activated DNase (ICAD) and poly(ADP)ribose polymerase (PARP) proteins to trigger apoptosis.

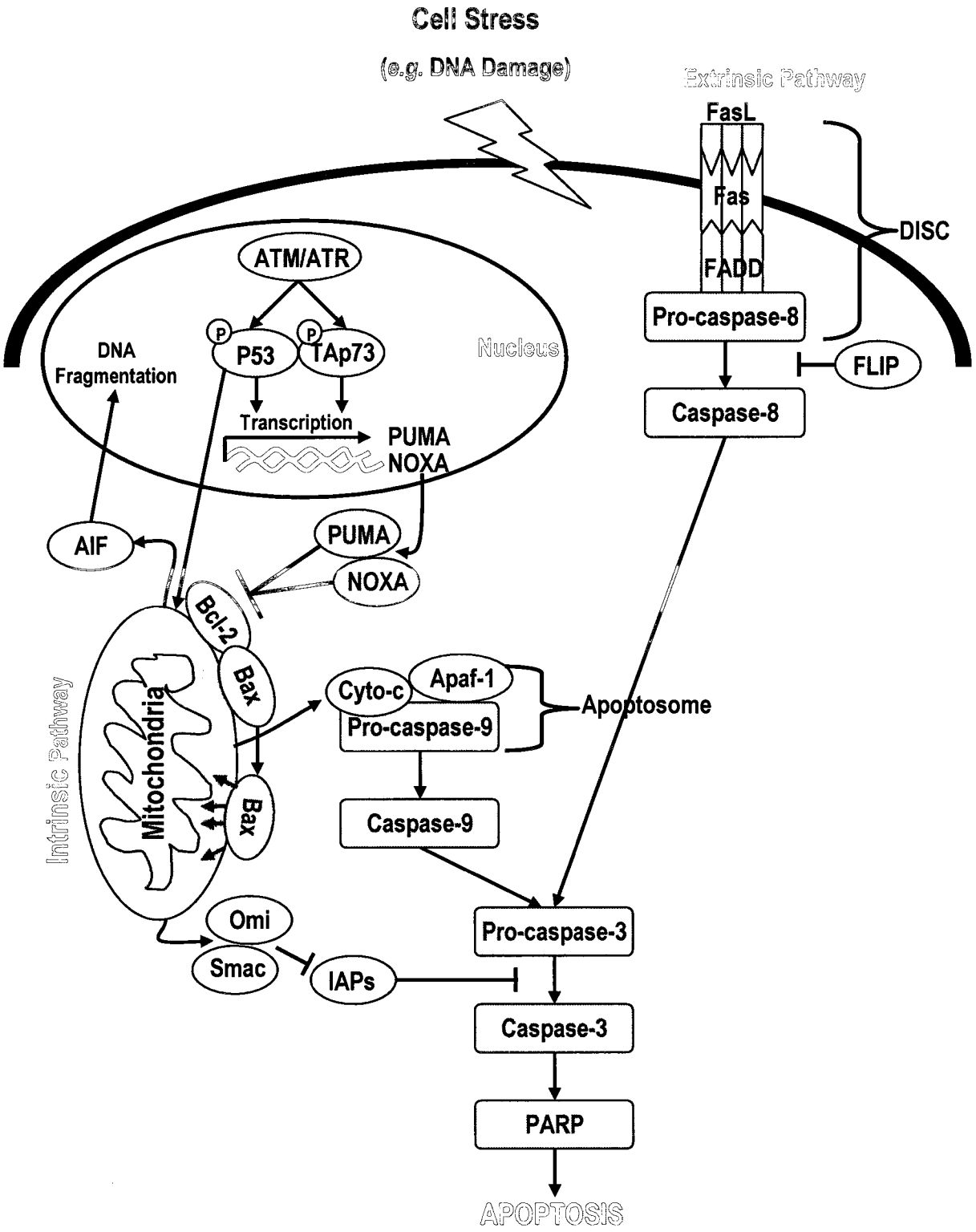

Cell Stress
(e.g. DNA Damage)
Extrinsic Pathway
FasL
Fas
FADD
DISC
Pro-caspase-8
FLIP
Caspase-8
ATM/ATR
P
P53
P
TAp73
Nucleus
DNA
Fragmentation
Transcription
PUMA
NOXA
AIF
PUMA
NOXA
Bcl-2
Bax
Cyto-c
Apaf-1
Apoptosome
Pro-caspase-9
Caspase-9
Intrinsic Pathway
Mitochondria
Bax
Omi
Smac
IAPs
Pro-caspase-3
Caspase-3
PARP
APOPTOSIS

Figure 1.2

1.4 CALCIUM HOMESTASIS

Calcium (Ca^{2+}) is the most abundant mineral in the human body. Many cellular processes are activated or inhibited by cellular signaling pathways through Ca^{2+}-dependent proteins. These processes include muscle contraction, synaptic transmission, cell proliferation and apoptosis (Berridge, Bootman *et al.* 2003). Ca^{2+} concentration and subcellular locations are firmly controlled, and alterations often result in pathological outcomes. Several studies have shown that some Ca^{2+}-mediated pathways are implicated in tumourgenesis and tumour progression through mechanisms involved in metastasis and invasion (Amuthan, Biswas *et al.* 2002; Huang, Kindzelskii *et al.* 2004; Liao, Schneider *et al.* 2006).

1.4.1 Calcium Signaling in Normal Cells

In the normal cell, the resting cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) is maintained at low levels (100 nM), in comparison to the extracellular free Ca^{2+} (1.2mM) (Carafoli 1987). Ca^{2+} is highly regulated in the intracellular compartments that act as Ca^{2+} stores, such as the sarcoplasmic/endoplasmic reticulum (SR/ER) (Monteith, McAndrew *et al.* 2007). The ER is an extensive network of folded membranes that is responsible for the production and transportation of several protein and lipid components of the cellular organelles. ER is the largest organelle used by the cell for Ca^{2+} storage and is enriched with Ca^{2+}-binding proteins that participate in Ca^{2+} signaling pathways. The most ubiquitous Ca^{2+}-binding protein is calmodulin, which mediates cellular processes such as apoptosis (Ahn, Lim *et al.* 2004).

Ca^{2+} release is regulated by specific membrane-associated proteins. There are three major classes of these proteins: channels, ATPases (pump) and exchangers (Carafoli 2002). Ca^{2+} channels allow Ca^{2+} diffusion down its concentration gradient, as with the Inositol 1,4,5-trisphosphate (IP_3) – and ryanodine-activated Ca^{2+} channels found on the ER membrane, or the voltage-gated Ca^{2+} channels, which are located at the plasma membrane. In contrast, Ca^{2+} pumps actively transport Ca^{2+} against its concentation gradient. This is examplified by the SR/ER Ca^{2+}ATPases (SERCAs) that pump Ca^{2+} into the ER (Monteith, McAndrew *et al.* 2007). As a Ca^{2+} exchanger, such as the plasmalemmal sodium ion (Na^+)/ Ca^{2+} exchanger, Na^+ is used to transport Ca^{2+} into the cell (Carafoli 2002).

1.4.2 Calcium Signaling in Cancer Cells

Ca^{2+}-mediated signaling pathways can be remodelled or deregulated in cancer cells. The remodelling or deregulation of these pathways can be due to epigenetic changes (*i.e.* methylation) in gene expression and/or post-translational changes in the regulators of such pathways. It has been shown that changes in the expression or activity of a Ca^{2+} channel or pump can have a promoting role in cancer. For example, in prostate cancer, an increase in the $[Ca^{2+}]_c$ is caused by the up-regulation of the transient receptor potential ion channel (TRPM6 and 8) at the plasma membrane (Tsavaler, Shapero *et al.* 2001). Likewise, the down-regulation of SERCA3 in colon cancer results in high $[Ca^{2+}]_c$ levels (Gelebart, Kovacs *et al.* 2002). Such an increase stimulates the Ca^{2+}-regulated proliferative pathways, thus offering growth advantages for cancerous cells.

It has been reported that Ca^{2+} channel localization can be altered in cancer cells, which could change the nature of Ca^{2+} signaling and lead to atypical biological responses. One study demonstrated that the human prostate cancer cell line, LNCaP, responds differentially to the cold/menthol stimulus than the normal prostate cells due to the mislocation of TRPM8 at the ER instead of the plasma membrane, resulting in a transient increase in Ca^{2+}, rather than a sustained Ca^{2+} inflow after activation (Thebault, Lemonnier *et al.* 2005).

A few studies have demonstrated mutations in genes regulating Ca^{2+}-mediated pathways. For example, somatic and germline mutations in lung cancer and germline mutations in colon cancer of *ATP2A2* result in the loss or reduction of SERCA2 expression (Korosec, Glavac *et al.* 2006). Collectively, these findings suggest that modulating Ca^{2+} signaling in cancer cells could be a useful theraputic target.

1.4.3 Calcium Signaling in Apoptosis

It is well documented that Ca^{2+} plays an important role in the activation and execution of apoptosis, where the increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is required for this process (Gerasimenko, Gerasimenko *et al.* 2002; Miyamoto, Howes *et al.* 2005). It has been reported that Ca^{2+} released from the ER could be a potent death-inducing signal. A direct link between IP_3R expression and activation and the induction of apoptosis has been found, where cells with reduced IP_3R level exhibited significantly less apoptosis (Jayaraman and Marks 1997).

Many studies have demonstrated a role for the mitochondria in the regulation of Ca^{2+}-mediated apoptosis and its cooperation with the ER related proteins (Miyamoto, Howes et al. 2005). It has been observed in HeLa cells that Ca^{2+} released from the ER upon ceramide treatment is loaded into the mitochondria for subsequent cytochrome c release. These responses could be prevented by Bcl-2 expression or by lowering $[Ca^{2+}]_{ER}$ and, thus apoptosis is attenuated (Pinton, Ferrari *et al.* 2001).

It has been also shown that the increase in $[Ca^{2+}]_i$ can activate certain cytosolic death effectors. This is especially relevant for calpains, which are potent initiators of apoptosis through the activation of caspases (Nakagawa and Yuan 2000) and other apoptotic factors, such as Bcl-2 (Gil-Parrado, Fernandez-Montalvan *et al.* 2002).

1.5 THE CALPAIN FAMILY

Calpains are a family of Ca^{2+}-dependent, non-lysosomal cysteine proteases that are widely expressed as ubiquitous and tissue-specific isoforms. They have been implicated in many basic cellular processes, including cell proliferation, apoptosis and differentiation (Perrin and Huttenlocher 2002). These processes are regulated by calpains through their protealytic activity on a wide range of cellular proteins, such as those of the cytoskeleton, structural and signaling proteins, membrane receptors and transcription factors. The calpain system consists of the calpain protease, a small regulatory subunit and the endogenous calpain inhibitor, calpastatin.

1.5.1 Calpain Structure

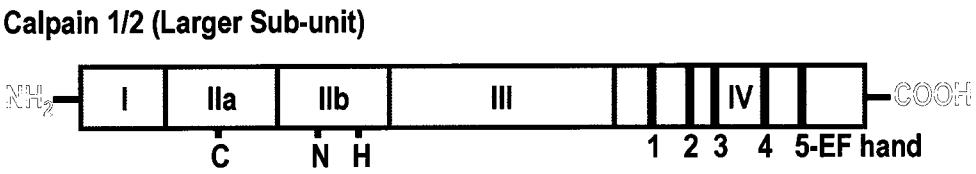
There are at least fifteen isoforms of calpain. The most well characterized are μ-calpain (Calpain 1) and m-calpain (Calpian 2), which are activated by micromolar (3-50 μM) and millimolar (0.4-0.8 mM) [Ca^{2+}] *in vitro* respectively (Carragher 2006). Each isoform functions as a heterodimer, consisting of a large catalytic subunit (Calpain 1/2) and a small regulatory subunit (Calpain 4). The larger subunit is subdivided into four domains based upon amino acid sequences (Figure 1.3) (Perrin and Huttenlocher 2002). These are: the short NH_2-terminal domain (I), the catalytic protease domain (II), the C2-like domain (III) and the Ca^{2+}-binding domain (IV). The small regulatory subunit consists of only two domains, domain V and domain VI, which are involved in binding to the large subunit.

Figure 1.3: Large catalytic and small regulatory subunits of the calpain structure.

Adapted from (Carragher 2006)

Both μ-calpain and m-calpain are heterodimers composed of unique catalytic subunits encoded by calpain 1 and calpain 2, respectively. They combine with the small regulatory subunit encoded by calpain 4. The large catalytic subunit is divided into four separate domains, determined by amino acid sequences. Domain I is a short NH-terminal, while domain II represents a distinct cysteine protease domain containing an active site of cysteine (C), histidine (H) and asparagine (N) residues. Domain III interacts with phospholipids and calcium. Domain IV contains five EF-hand (Helix-loop-helix structural domain) motifs. The first four act as calcium binding sites, while the fifth is involved in dimerization with the regulatory domain of calpain 4.

The small regulatory subunit consists of two domains: a glycine rich domain V, currently with no known function; and domain VI, which contains five EF-hand motifs, where the fifth motif binds with domain IV of the large subunit.


Calpain 1/2 (Larger Sub-unit)
NH2
I
IIa
IIb
III
IV
COOH
C
N H
1 2 3 4 5-EF hand

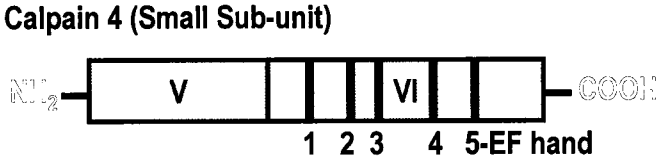

Calpain 4 (Small Sub-unit)
NH2
V
VI
COOH
1 2 3 4 5-EF hand

Figure 1.3

1.5.2 Regulation of Calpain Activity

Calpain is normally abundant in the cytoplasm and the spatial regulation is necessary for its activation. It is usually regulated post-transcriptionally by different mechanisms, including inhibition by calpastatin, activation by Ca^{2+} and autoproteolytic cleavage. Regarding calpastatin regulation, the calpain:calpastatin ratio normally varies between tissue; however, calpastain is generally found at much higher levels than calpain in cancerous cells. The modulation of this ratio and/or the sub-cellular translocation of either calpastatin or calpain have been shown to regulate calpain activity (Lane, Allan *et al.* 1992; Tullio, Passalacqua *et al.* 1999).

The Ca^{2+} concentration required for calpain activation is generally above the physiological level of Ca^{2+} found in normal cells. Certain stimuli such as pharmacological agents (*i. e.* Thapsigargin or Cyclopiazonic acid (CPA)) causing ER-induced stress and the elevation of $[Ca^{2+}]_i$, can influence calpain activation (Harriman, Liu *et al.* 2002).

Calpain can also be regulated by phosphorylation (Shiraha, Glading *et al.* 1999; Glading, Chang *et al.* 2000; Glading, Uberall *et al.* 2001). Epidermal growth factors (EGF) activate calpain via an ERK/MAP kinase pathway (Glading, Chang *et al.* 2000; Glading, Uberall *et al.* 2001). ERK directly phosphorylates m-calpain and increases its proteolytic activity (Shiraha, Glading *et al.* 1999). Furthermore, the intracellular distribution of calpain may be an important mechanism to regulate its activity. Active calpain is present predominantly at the plasma membrane in T-cells stimulated with fibronectin (Rock, Dix *et al.* 2000).

1.5.3 Calpain-Mediated Apoptosis

It is well known that calpain is involved in the regulation of apoptosis. Whether it promotes or suppresses apoptosis remains unresolved. It has been reported that calpain acts as a pro-apoptotic protein through the activation of caspase 3 and 12 (Nakagawa and Yuan 2000; McCollum, Nasr *et al.* 2002) and the cleavage of Bad and Bid proteins (Wood, Thomas *et al.* 1998; Wood and Newcomb 2000; Mandic, Viktorsson *et al.* 2002). Calpain induces apoptosis in murine neuronal cells by activating p53 (Chua, Guo *et al.* 2000; Sedarous, Keramaris *et al.* 2003). In contrast, calpain has been shown to suppress apoptosis through the cleavage of p53 (Atencio, Ramachandra *et al.* 2000) and caspase 7 and 9 (Chua, Guo *et al.* 2000; Sedarous, Keramaris *et al.* 2003). These conflicting reports suggested that the role of calpain in the regulation of apoptosis may be dependent upon cell-type and/or the nature of the apoptotic stimulus involved. Further investigations are necessary to fully understand these parameters and to establish the exact role of calpain in apoptosis in different cellular contexts.

1.6 THE P53 FAMILY

The p53 family is a unique intracellular system that mediates and links the pathways of cell fate determination (*i.e.* proliferation, differentiation or apoptosis) (Danilova, Sakamoto *et al.* 2008). It is composed of three genes: *P53*, *P63* and *P73,* located at chromosome 17, 3 and 1, respectively. The *P63* and the *P73* genes encode two primary transcripts that are each controlled by two separate promoters: P1 which generates the full length protein and P2, which produces truncated proteins lacking part of the N-terminal domain (Figure 1.4). The full length proteins of the p53 family consist of the transactivation (TA) domain, the DNA-binding (DBD) domain and the

oligomerization domain (OD) (Harms and Chen 2006). The p63 and p73 proteins are supplemented to contain an extra C-terminus domain, known as sterile alpha motif (SAM); it is involved in protein-protein interactions (Pietsch, Sykes *et al.* 2008). Complexity is added to the family by the alternative splicing at the C-terminus, giving rise to several splice variants for the p63 (α, β and γ) and the p73 protein members (α, β, γ, δ, ε, ζ and η). *P73* gene is further complicated by the addition of four alternatively spliced N-terminal isoforms. The *P73* gene expresses at least 35 mRNA variants, theoretically encoding twenty-nine different protein isoforms. Currently, only fourteen different protein isoforms have been characterized (Murray-Zmijewski, Lane *et al.* 2006). Although the p53 family members share structural and functional qualities, each has distinct regulation, and thus, different activity. The members of p53 family are mutually dependent and operate cooperatively.

1.6.1 P53

P53 is best described as 'guardian of the genome.' P53 is a tumour suppressing protein. It has been intensively studied since its discovery in the late 1970s (Lane and Crawford 1979). P53 integrates cellular signals controlling cell survival and death and it is activated by stresses such as DNA-damage and oncogenic stress. Depending upon the severity of the stress stimuli, p53 can either inhibit cell proliferation by cell cycle arrest or eliminate the cell by initiating apoptosis (Soussi 2003).

P53 content is normally kept low by murine double-minute 2 (MDM2), which targets p53 for proteasomal degradation (Honda, Tanaka *et al.* 1997). Upon activation by cellular stress, p53 is up-regulated and stabilized by different means of post-translational modifications, (mainly by phosphorylation). Subsequently, it induces the transcriptional

activation of several down-stream genes implicated in both cell-cycle arrest (p21) and apoptosis (PUMA and NOXA).

A significant role of p53 in the regulation of ovarian cancer cell sensitivity to CDDP-induced apoptosis has been established, thereby uncovering some of the p53-related mechanisms underlying chemoresistance in these cells. Certain studies have shown that CDDP induces p53 phosphorylation, and thus stabilization in chemosensitive ovarian cancer cells (Shimada, Kigawa et al. 2000; Fraser, Leung et al. 2003; Horiuchi, Wang et al. 2007; Fraser, Bai et al. 2008). P53-mediated PUMA up-regulation and apoptosis induction has also been demonstrated in these cells. The PUMA up-regulation and apoptosis has not been exhibited in the chemoresistant cells by p53. Once p53 is activated, it induces cytochrome c, Smac and AIF release from the mitochondria in chemosensitive cells (Yang, Fraser *et al.* 2006; Yang, Fraser *et al.* 2008). These effects are attenuated in the chemoresistant cells by an Akt-dependent pathway.

In addition, p53 has been found to facilitate FLIP down-regulation by CDDP-induced FLIP ubiqutination and proteasomal degradation (Abedini, Muller *et al.* 2008). In a p53-dependent manner, both PTEN (phosphatase and tensin homologue) over-expression (Lee, Choi et al. 2005; Yan, Fraser et al. 2006) and the Nitric oxide (NO) donor S-nitroso-N-acetylpenicillamine (SNAP) (Leung, Fraser *et al.* 2008) enhances CDDP-induced apoptosis in ovarian cancer cells.

The p53-mediated mechanisms that were deregulated in chemoresistant ovarian cancer cells due to the action of certain survival factors have been determined. Basal soluble guanylyl cyclase (sGC) activity and cyclic guanosine monophosphate (cGMP) regulates p53 protein stability, content, and its function. It is possible that cell survival can

be attributed to the altering of p53 phosphorylation and stabilization, thereby promoting cell survival in chemoresistant ovarian cancer cells (Fraser, Chan *et al.* 2006).

It has been demonstrated that Xiap (Fraser, Leung et al. 2003; Mansouri, Zhang et al. 2003) and Akt (Yang, He et al. 2006; Fraser, Bai et al. 2008) promote CDDP resistance through the inhibition of p53 phosphorylation and thus, its transcriptional activity in ovarian cancer cells. Also, Akt confers CDDP resistance by modulating CDDP-induced, p53-dependent FLIP ubiquitination (Abedini, Muller *et al.* 2009). These significant findings suggest that p53 plays a vital role in regulating CDDP sensitivity in ovarian cancer cells by modulating pathways involved in CDDP-induced apoptosis.

1.6.2 P63

P53 was believed to be a unique member of its own until the discovery of *P73* and *P63* genes (Kaghad, Bonnet *et al.* 1997; Yang, Kaghad *et al.* 1998). Unlike the *P53* gene, which is mutated in 50% of ovarian cancers (Leitao, Soslow *et al.* 2004), *P63* and *P73* are rarely mutated in ovarian cancer (Muller, Schleithoff *et al.* 2006). It has been shown that the TAp63 protein may be involved in the development of benign and malignant epithelial ovarian tumours, whereas ΔNp63 expression has been associated with poor survival in ovarian cancer patients (Poli Neto, Candido Dos Reis *et al.* 2006; Marchini, Marabese *et al.* 2008).

Similar to p53, TAp63 is up-regulated by DNA-damaging agents through post-translational modifications. In turn, transcriptional activation of certain p53-responsive targets such as CD95 are induced (Katoh, Aisaki *et al.* 2000; Okada, Fukasawa *et al.* 2002). Some of these modifications (*i.e.* serine phosphorylation) affecting TAp63 activity have been identified (Petitjean, Ruptier *et al.* 2008); however, the specific influence on its apoptosis function is not clear. In contrast to the TAp63 isoforms, the ΔNp63 isoforms negatively regulate apoptosis through the induction of heat shock protein 70 (HSP70), which is anti-apoptotic protein (Wu, Nomoto *et al.* 2003).

1.6.3 P73

The human *P73* gene is composed of 14 exons spanning 80,000 base pairs (bp) on chromosome 1p36.3 (Ozaki, Hosoda *et al.* 2005). Despite its significant homology to p53, *P73* is not a classical Knudson-type tumour suppressor gene (Melino, Lu *et al.* 2003; Moll 2003; Moll and Slade 2004). In addition to its rare mutation rate, *P73* gene does not show a significant loss of heterozygosity in ovarian cancer (Hu, Ulaner *et al.* 2000). It has been determined that ΔNp73 is up-regulated in ovarian tumours, suggesting that its expression might play an important role in the pathogenesis of ovarian cancer (Ng, Yiu *et al.* 2000; Zaika, Slade *et al.* 2002).

The relative expression of the different p73 isoforms is not well characterized and several studies revealed that the expression of p73 isoforms might be tissue-specific. For example, it has been reported that ΔNp73 is over-expressed in tumours of the lung, breast, brain, colon, ovary, thymus and prostate but not in their related healthy tissues (Uramoto, Sugio et al. 2004; Guan and Chen 2005; Dominguez, Garcia et al. 2006). Others demostrated that both TAp73 and ΔNp73 isoforms were upregulated in cervical

squamous cell carcinoma and myosarcoma (Becker, Pancoska et al. 2006; Liu, Chan et al. 2006).The expression of the TAp73 isoforms has been shown to be regulated by certain transcription factors such as E2Fs (Ozaki, Okoshi et al. 2009) while the expression of ΔNp73 isoforms is regulated by its own members; TAp73 and p53 (Stiewe 2007; Danilova, Sakamoto et al. 2008).

Figure 1.4: Structure of the p53 family

Adapted from (Murray-Zmijewski, Lane et al. 2006)

The p53 family is composed of three genes: *P53*, *P63* and *P73,* located at chromosome 17, 3 and 1, respectively. The *P63* and the *P73* genes encode two primary transcripts that are each controlled by two separate promoters: P1 which generates the full length protein and P2, which produces truncated proteins lacking part of the N-terminal domain. The full length proteins of the p53 family consist of the transactivation (TA) domain, the DNA-binding domain and the oligomerization domain (Oligo). *P63* and *P73* genes contain an extra C-terminus domain, known as sterile alpha motif (SAM); it is involved in protein-protein interaction. Alternative splicing at the C-terminus also gives rise to several splice variants for the p63 (α, β and γ) and the p73 members (α, β, γ, δ, ε, ζ and η). *P73* gene is further complicated by the addition of four alternatively spliced N-terminal isoforms: including: Ex2p73, Ex2/3p73, ΔN'p73 and ΔNp73. Ex2p73 arises from alternative splicing of exon 2 and only contains part of this domain. Ex2/3p73 arises from alternative splicing of both exon 2 and 3. It is initiated from exon 4, and therefore lacks the entire TA domain. ΔN'p73 results from alternative splicing of exon 3' contained in intron 3. Finally, ΔNp73 encoded from promoter P2 lacks the TA domain, but it contains a unique 13 amino acid chain.

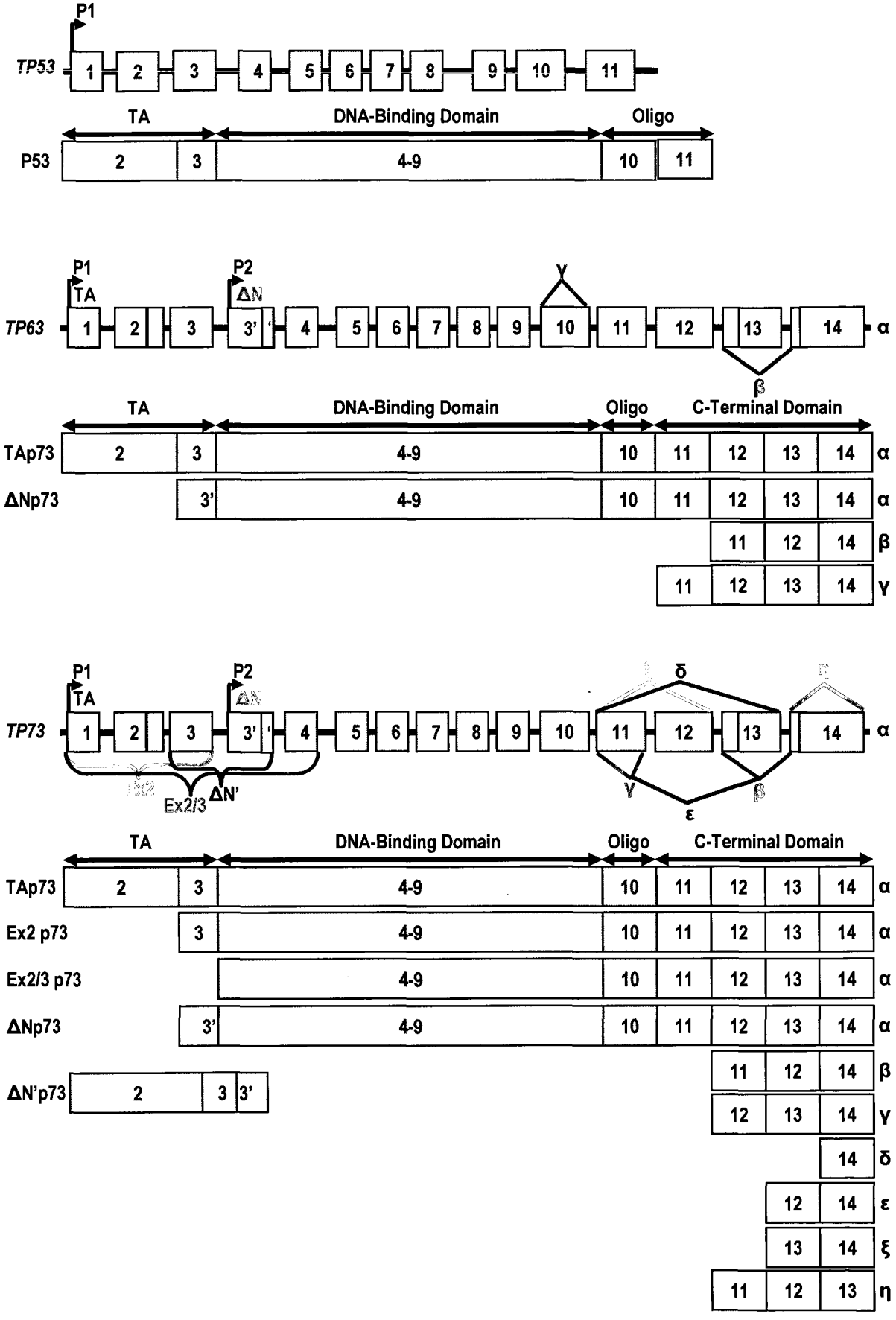

P1
TP53
1 2 3 4 5 6 7 8 9 10 11
TA
DNA-Binding Domain
Oligo
P53
2 3 4-9 10 11
P1
TA
P2
ΔN
γ
TP63
1 2 3 3' 4 5 6 7 8 9 10 11 12 13 14 α
β
TA
DNA-Binding Domain
Oligo
C-Terminal Domain
TAp73
2 3 4-9 10 11 12 13 14 α
ΔNp73
3' 4-9 10 11 12 13 14 α
11 12 14 β
11 12 13 14 γ
P1
TA
P2
ΔN
δ
TP73
1 2 3 3' 4 5 6 7 8 9 10 11 12 13 14 α
Ex2/3
ΔN'
γ
β
ε
TA
DNA-Binding Domain
Oligo
C-Terminal Domain
TAp73
2 3 4-9 10 11 12 13 14 α
Ex2 p73
3 4-9 10 11 12 13 14 α
Ex2/3 p73
4-9 10 11 12 13 14 α
ΔNp73
3' 4-9 10 11 12 13 14 α
ΔN'p73
2 3 3'
11 12 14 β
12 13 14 γ
14 δ
12 14 ε
13 14 ξ
11 12 13 η

Figure 1.4

1.6.3.1 P73-induced Apoptosis

Several studies have demonstrated an important role of TAp73 in the induction of apoptosis. P73 is activated in response to genotoxic agents, such as DNA-damaging agents (Agami, Blandino *et al.* 1999; Gong, Costanzo *et al.* 1999; Zaika, Irwin *et al.* 2001; Irwin, Kondo *et al.* 2003). Upon activation, TAp73 acts as a transcription factor that initiates cell cycle arrest and apoptosis through the activation of putative p53 target genes (Melino, Bernassola *et al.* 2004; Muller, Schilling *et al.* 2005). TAp73 also activates unique downstream targets such as transmembrane proteins JAG, suggesting a role that is distinct from p53 (Fang, Lee *et al.* 1999; Fontemaggi, Kela *et al.* 2002).

The role of ΔNp73 in apoptosis has not been established.yet. Several studies have found that ΔNp73 acts as a dominant negative regulator of TAp73 and p53. This is either by hetero-oligomerization with p73 (Grob, Novak *et al.* 2001; Nakagawa, Takahashi *et al.* 2002) or p53 (Kartasheva, Contente *et al.* 2002) or by competitive inhibition of the binding to p53-responsive genes (Stiewe, Theseling *et al.* 2002). Other reports have demonstrated that ΔNp73 contains a unique thirteen amino acid, crucial for the expression of various genes, such as heat shock protein 70 (HSP 70) and nuclear factor kappa B (NFκB) (Tanaka, Kameoka *et al.* 2004; Tanaka, Ota *et al.* 2006). ΔNp73β and ΔNp73γ have been found to be active in transactivation and growth suppression (Liu, Nozell *et al.* 2004). Instances of forced expression of ΔNp73α fail to affect cell growth or to increase resistance to anticancer drug; it does not antagonize p53 apoptotic functions (Sabatino, Previdi *et al.* 2007; Marabese, Marchini *et al.* 2008).

When considered together, these observations suggest that ΔNp73 may play a cell-type specific role, or may interact with additional mediators, resulting in differential phenotypes depending upon the type of cell. Further work is required to precisely elucidate the role of ΔNp73 in drug-induced apoptosis in cancer cells.

P73 activation is modulated by post-translational modifications (Figure 1.5), which have been shown to play a major role in its apoptotic function. Endogenous p73 is both activated and stabilized in the DNA damage-induced apoptotic response (γ-irradiation and CDDP treatment) by non-receptor tyrosine kinase c-Abl via tyrosine phosphorylation (Agami, Blandino *et al.* 1999; Gong, Costanzo *et al.* 1999). Members of the mitogen-activated protein kinase (MAPK) superfamily were shown to regulate p73 activity in CDDP-induced apoptosis. P38 MAP kinase has been reported to phosphorylate p73 at the threonine residues adjacent to proline in human embryonic kidney 293 (HEK 293) cells, thus enhancing its transcriptional activity (Sanchez-Prieto, Sanchez-Arevalo *et al.* 2002). Another member of the MAP kinases is JNK (c-Jun N-terminal kinase), which also phosphorylates p73 at C-terminal domain (Ser412, Thr442 and Thr482) in response to CDDP. This results in its activation and thus apoptosis induction in non-small cell lung carcinoma (H1299) cells (Jones, Dickman *et al.* 2007). Reacting to the presence of CDDP, Chk1 phosphorylates p73 at serine 47 and enhances its transcriptional activity and apoptotic function (Gonzalez, Prives *et al.* 2003). CDDP promotes a complex formation between p73 and a protein kinase Cδ catalytic fragment (PKCδ); it phosphorylates p73 at serine 289, increasing both its stability and transcriptional activity (Ren, Datta *et al.* 2002).

On the other hand, p73 can be negatively regulated by post-translational modifications, which in turn inhibit its function. It has been reported that the Cyclin-dependent kinases (CDK) phosphorylate p73 at threonine 86, where such phosphorylation attenuated the transcriptional activity of p73 (Gaiddon, Lokshin *et al.* 2003).

Figure 1.5: Post-translational modifications of p73 protein induced by CDDP.

Adapted from (Ozaki and Nakagawara 2005).

During CDDP-induced apoptosis, p73 is phosphorylated by c-abl (Tyr-99), Chk1 (Ser-47), PKCδ (Ser-289) and JNK kinase (Ser412, Thr442 and Thr482). The phosphorylation increases p73 stability, thus enhancing its transcriptional activity in the up-regulation of pro-apoptotic genes (indicated in Figure 1.2).

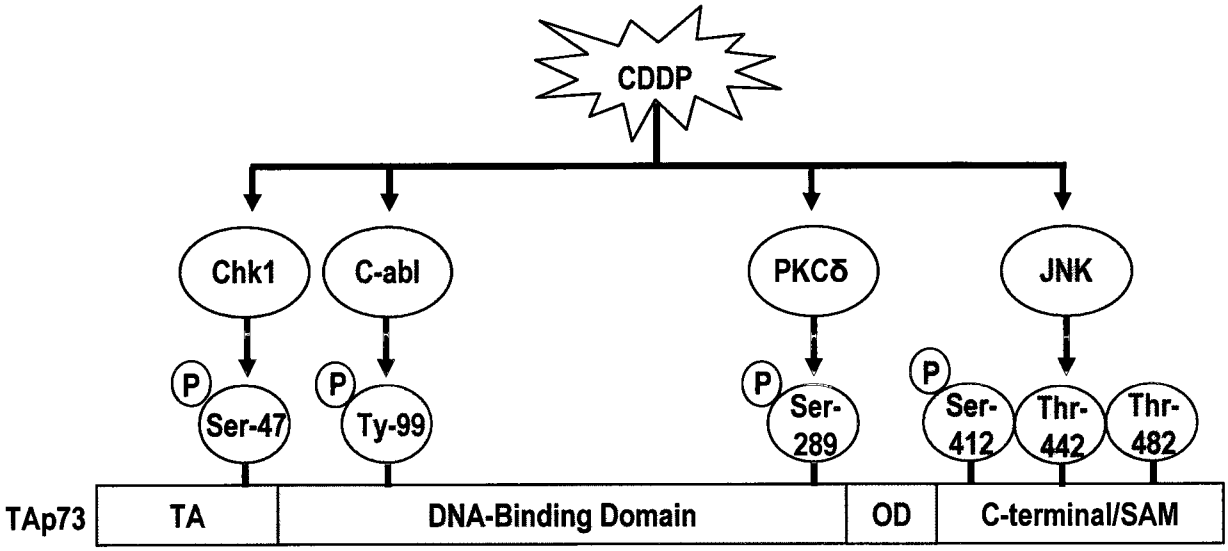

CDDP
Chk1
C-abl
PKCδ
JNK
P
Ser-47
P
Ty-99
P
Ser-289
P
Ser-412
Thr-442
Thr-482
TAp73
TA
DNA-Binding Domain
OD
C-terminal/SAM

Figure 1.5

1.6.3.2 P73 and Chemosensitivity

Several reports have linked the chemosensitivity of cancer cells to p73 function. Inactivation of TAp73 by dominant negative expression or RNA interference has lead to resistance and attenuation of drug-induced apoptosis (Irwin, Kondo *et al.* 2003). TAp73 over-expression significantly sensitises a subset of melanoma cell lines to Adriamycin- and CDDP-induced apoptosis (Tuve, Racek *et al.* 2006). The down-regulation of ΔNp73 by anti-sense techniques enhances p53/TAp73-mediated apoptosis in cancer cells in response to chemotherapeutic drugs, while its over-expression confers chemoresistance (Ishimoto, Kawahara *et al.* 2002; Zaika, Slade *et al.* 2002). Linking these findings is the postulate that p73 could be a determinant of chemosensitivity in certain cancer cells by its vital role in drug-induced apoptosis. Understanding precisely the differential role of both the TA and the ΔN isoforms of p73 and their interaction with the other family members will improve the current understanding of the molecular and cellular basis of chemoresistance.

1.6.3.3 P73 Turnover

Recently, p73 turnover as a topic of research has emerged. Unfortunately, very few studies have yet examined the mechanisms that regulate p73 degradation. Preliminary studies have shown p73 function to be regulated by its protein stability. This is through the biochemical pathways of proteasome-, caspase- and calpain-dependent pathways.

Normally, the cellular p73 content is kept low by rapid degradation via the ubiquitin-dependent and -independent proteasome pathways (Bernassola, Salomoni *et al.* 2004; Rossi, De Laurenzi *et al.* 2005). The ubiquitin-dependent pathway is regulated by

Itch, a NEDD4-like Hect-E3 ubiqutin ligase (Rossi, De Laurenzi *et al.* 2005). The ubiquitin-independent pathway is controlled by the NAD(P)H quinine oxidoreductase 1 (NQO1) (Asher, Tsvetkov *et al.* 2005). Both of these pathways physically interact with p73 as a target for proteasomal degradation.

Studies have demonstrated the differential regulation of the steady-state level of TAp73 and ΔNp73 proteins. This may be attributed to the differential function of these proteins in cellular processes including apoptosis. As an example, the ΔNp73 is rapidly degraded upon DNA-damage, facilitating p53/TAp73-mediated apoptosis in osteosarcoma cells (Maisse, Munarriz *et al.* 2004).

The involvement of caspase in regulating the steady-state level of p73 is a very new finding and only one published study has addressed this issue. This study demonstrated that p73 is cleaved by caspase-3 and caspase-8 during induction of apoptosis by DNA-damaging drugs and by TRAIL receptor ligation (Sayan, Sayan *et al.* 2008). TAp73 and some of its cleavage products are localized at the mitochondria. TAp73 down-regulation significantly attenuated TRAIL-induced apoptosis in HCT116 cells suggesting that it is required for TRAIL-mediated apoptosis.

Calpain has been shown to regulate the steady state of p73 levels (Munarriz, Bano et al. 2005). It cleaves TAp73 and ΔNp73 isoforms with the C-terminus variant, such as α, β and γ. Calpain inhibition, either by the endogenous (Calpastatin) or synthetic (Calpeptin) inhibitors, increases the steady-state p73 protein level. This finding indicates a regulatory role of calpain in p73 stability. These observations should serve as platform for additional investigation, in order to fully delineate the mechanisms involved in regulating p73 content in drug-induced apoptosis in cancer cells.

CHAPTER 2: OBJECTIVES AND HYPOTHESES

Despite the progress made in our understanding of tumour biology and the improvement in treatment strategies, the long-term outcome of ovarian cancer (OVCA) treatment remains poor. Ovarian cancer is the most lethal cancer among those of gynecological origin (Canadian Cancer Society, 2009). Poor prognoses are explained, partially, by late diagnosis and chemoresistance, as previously discussed. CDDP resistance is a multi-factorial event and involves changes in the expression of various protein factors contributing to CDDP-induced apoptosis. The discovery and modulation of these proteins will potentially enable us to detect and overcome CDDP resistance in ovarian cancer.

Our lab has extensively investigated some of the proteins that are involved in the regulation of ovarian cancer cell sensitivity to CDDP and how the deregulation of these pathways might contribute to chemoresistance. Specifically, we have shown that certain cell survival factors (*i. e.* Akt, Xiap and FLIP) are important determinants of CDDP resistance, attenuating CDDP-induced apoptosis in ovarian cancer (Asselin, Mills *et al.* 2001; Fraser, Leung *et al.* 2003; Abedini, Qiu *et al.* 2004; Yang, Fraser *et al.* 2006; Abedini, Muller *et al.* 2008; Fraser, Bai *et al.* 2008; Abedini, Muller *et al.* 2009). We have also studied the involvement of essential pro-apoptotic factors that enhance ovarian cancer cell sensitivity to CDDP, by the regulation of its apoptotic effect on p53, PTEN and AIF (Fraser, Chan *et al.* 2006; Yan, Fraser *et al.* 2006; Fraser, Bai *et al.* 2008; Yang, Fraser *et al.* 2008).

Since its discovery in 1997 (Kaghad, Bonnet *et al.* 1997), p73 has emerged as a vital regulator of the drug-induced apoptosis of cancer cells (Agami, Blandino *et al.* 1999; Di Como, Gaiddon *et al.* 1999; Fang, Lee *et al.* 1999). P73 is considered a viable target that could replace p53, especially in p53-mutant or -null cancer cells. Due to its transcriptional properties that resemble the function of p53, p73 is a compatible target with the additional advantage of being rarely mutated in cancer cells (Hu, Ulaner *et al.* 2000). The role of p73 in the regulation of chemosensitivity in ovarian cancer is poorly understood, particularly how deregulation of p73-mediated apoptosis confers resistance to CDDP treatment remains unclear. Elucidating the mechanism(s) by which p73 may regulate CDDP-induced apoptosis could be essential in determining the underlying mechanisms of CDDP resistance. We believe that therapeutic strategies designed for p73 activation are a promising approach for the treatment of ovarian cancer.

Although there are many isoforms of both TA-and ΔN-p73, the TAp73α and ΔNp73α isoforms were selected for the studies performed for the purposes of this thesis. This selection was based firstly upon the availability of specific p73α isoform-related reagents, such as antibodies, plasmids and small interference RNA (siRNA). Secondly, the α isoforms are representative of the full-length form of the p73 protein. They were chosen to parallel any distinct function of the full-length form, as opposed to selecting the other isoforms, which would lack some of the untranscribed exons, such as the absence of exon 13 as with p73β.

2.1 OVERALL OBJECTIVE

The overall objective of the present study was to examine the involvement of p73α in the cellular and molecular mechanisms of chemoresistance and its regulation by CDDP in ovarian cancer cells. Understanding the molecular basis of such involvement may provide clues for developing novel strategies for the treatment of chemoresistant ovarian cancer.

2.2 OVERALL HYPOTHESIS

P73α plays an essential role in the regulation of ovarian cancer cell sensitivity to CDDP-induced apoptosis.

2.3 SPECIFIC HYPOTHESES

1. P73α is involved in CDDP-induced apoptosis.
2. CDDP-induced p73α processing is mediated by the calpain pathway.
3. The p73α-calpain interaction and cytoplasmic colocalization are a pre-requisite for CDDP-induced, calpain-mediated p73α processing.
4. Ca^{2+} is required for calpain activation and CDDP-induced apoptosis.
5. CDDP-induced increase in intracellular calcium concentration ($[Ca^{2+}]_i$) is due to mobilization of internal stores.
6. Ca^{2+}-dependent, calpain-mediated p73α processing is involved with CDDP-induced apoptosis in ovarian cancer cells.

2.4 SPECIFIC OBJECTIVES

1. To establish the role of TAp73α and ΔNp73α in CDDP-induced apoptosis in ovarian cancer cells. Specifically, to determine the effect of manipulating the TAp73α and the ΔNp73α content in CDDP-induced apoptosis by both down-regulation and over-expression strategies. To examine the effect of CDDP on the content of TAp73α and ΔNp73α in both chemosensitive and chemoresistant ovarian cancer cells.
2. To examine the underlying mechanism(s) by which CDDP regulates the TAp73α and the ΔNp73α content in ovarian cancer cells, including the effect on their mRNA and protein abundance, the involvement of the proteasome pathway and the calpain-dependent pathway.
3. To elucidate the contribution of Ca^{2+}-dependent, calpain activation to CDDP-mediated, TAp73α and ΔNp73α regulation in ovarian cancer cells. Specifically, to assess the effect of CDDP on the $[Ca^{2+}]_i$ in chemosensitive and chemoresistant ovarian cancer cells and how the deregulation of this pathway enhances CDDP-resistance.

Figure 2.1: A hypothetical model illustrating the role and regulation of p73α in CDDP-induced apoptosis in chemosensitive and chemoresistant OVCA cells.

In chemosensitive cells, CDDP increases the $[Ca^{2+}]_i$, resulting in calpain activation, thereby cleaving p73α. The cleaved-p73α then translocates to the nucleus where it is activated by CDDP-induced, DNA-damage. The activated p73α then casues transcriptional activation of PUMA and NOXA, which in turn translocates to the mitochondria. Subsequently, cytochrome c is released, activating caspase-3 and inducing apoptosis. This pathway is attenuated in chemoresistant cells due to the lack of Ca^{2+} release and of calpain activation and thus failure of p73α cleavage.

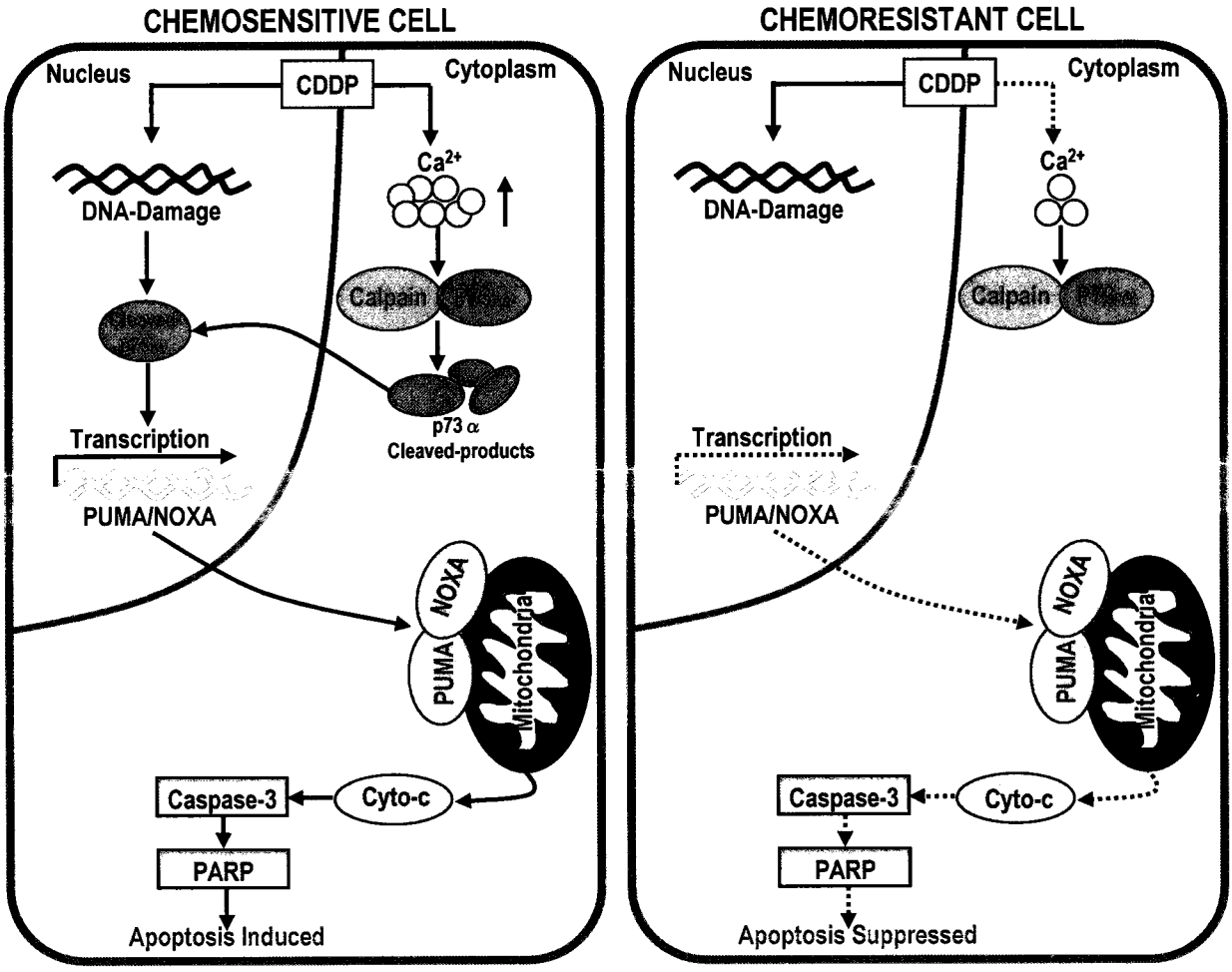

CHEMOSENSITIVE CELL
Nucleus
CDDP
Cytoplasm
DNA-Damage
Ca2+
Calpain
p73 α
Cleaved-products
Transcription
PUMA/NOXA
NOXA
PUMA
Mitochondria
Caspase-3
Cyto-c
PARP
Apoptosis Induced
CHEMORESISTANT CELL
Nucleus
CDDP
Cytoplasm
DNA-Damage
Ca2+
Calpain
Transcription
PUMA/NOXA
NOXA
PUMA
Mitochondria
Caspase-3
Cyto-c
PARP
Apoptosis Suppressed

Figure 2.1

CHAPTER 3: MATERIALS AND METHODS

3.1 Reagents

Cis-diaminedichloroplatinum (Cisplatin; CDDP), Dimethyl sulfoxide (DMSO), Hoechst 33258, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), aprotinin, and cycloheximide, 5-Aza Deoxycytidine were purchased from Sigma (St Louis, MO, USA). Proteasome inhibitors (Lactacystin and Epoxomicin) were from Calbiochem (San Diego, CA, USA). Calpain Inhibitor (Calpeptin) was purchased from Enzo Life Science International Inc. (Plymouth Meeting, PA, USA). The siRNA for p73α, calpain and $p34^{cdc2}$ and scrambled sequence siRNA (control) were provided by Ambion Inc. (Austin USA), Santa Cruz Biotechnologies (San Diego, CA, USA) and Dharmacon Inc. (Lafayette, CO, USA), respectively. RiboJuice and Lipofectamine Plus were from Novagen Inc. (San Diego, CA, USA) and Fluo4-AM dye, Cyclopiazonic acid (CPA) and Caffeine were from Invitrogen (Carlsbad, CA, USA). Ca^{2+} -free media (RPMI-1640) was purchased from United States Biological (Swampscott, MA, USA). Primary antibodies for Western blot were rabbit polyclonal anti-p73 (1:2000; (Sayan, Paradisi et al. 2005)), anti-PARP, anti-α-fodrin, anti-calpain and anti-phospho-threonine (1:5000; Cell Signalling Technology, Beverly, CA, USA), anti-PUMA (1:1000; Santa Cruz Biotechnologies), anti-Xiap (1:1000; Trevigen, Gaithersburg, MD, USA), mouse monocloncal anti-NOXA (1: 1000) and anti-GAPDH (ab8245, Abcam, Cambridge, UK), in addition to anti-p73 (GC-15) and anti-$p34^{cdc2}$ (1:1000; Cell Signalling Technology, Beverly, CA, USA), as well as rat anti-HA (1:1000; clone 3F10, Roche, Laval, Quebec, Canada) and goat anti-GRP78 (1:5000; Santa Cruz Biotechnologies) antibody.

Goat anti-p73 (C-17; Santa Cruz Biotechnologies) was used for p73α immunoprecipitation. The horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad (Hercules, CA, USA). Enhanced chemiluminescent reagents and films were purchased from Amersham Biosciences (Buckinghamshire, UK).

3.2 Cell culture

In the present thesis, five established human ovarian cancer cell lines were used: CDDP-sensitive human OVCA cells (OV2008 and A2780s) and their resistant isogenic counterpart (C13* and A2780cp, respectively), CDDP-sensitive (OVCA 432) and CDDP-resistant (Hey) cells. OV2008 and A2780s cells were established from serous cystodenocarcinoma of two different patients prior chemotherapy (DiSaia, Sinkovics *et al.* 1972) while their resistant variants were developed through extended culture in the presence of increasing concentrations of CDDP (Brown, Clugston *et al.* 1993; Mamenta, Poma *et al.* 1994). OVCA432 cells were also established from serous cystoadenocarcinoma (Elbendary, Berchuck *et al.* 1994) while Hey cells were from a moderately differentiated papillary cystadenocarcinoma (Buick, Pullano *et al.* 1985). All of the above cells harbour wild-type p53 excepting the A2780cp and the OVCA 432 cells that contain mutant p53. The OVCA cells were kindly provided by Dr. B. Vanderhyden at the Ottawa Regional Cancer Center.

OV2008, C13* and Hey cells were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) medium while A2780s, A2780cp and OVCA 432 were maintained in Dulbecco's modified Eagle (DMEM-F12) medium. The medium was supplemented with fetal bovine serum (10%), streptomycin (50 mg/ml), penicillin (50 U/ml), fungizone (0.625 g/ml; Life Technologies, Inc., BRL, Carlsbad, CA, USA), and non-essential amino

acids (1%), as previously reported (Abedini, Muller *et al.* 2008; Fraser, Bai *et al.* 2008; Abedini, Muller *et al.* 2009).

3.3 Transient transfection

All cell lines used (2.4 x 10^5) were seeded in 60 mm dishes and transfected the following day with 2 μg of pcDNA3.1-GFP vector alone, or pcDNA3.1 vectors containing TAp73α cDNA or ΔNp73α cDNA (Generous gift from Dr. Melino; University of Leicester, Leicester, UK), using the Lipofectamine Plus Transfection reagents (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, cells were treated with CDDP (10 μM) or DMSO (vehicle control) for another 24 h and then harvested for analysis.

3.4 Adenovirus infection

OV2008 cells were infected with adenoviral Xiap-sense or LacZ control (5 MOI) as reported previously (Fraser, Leung *et al.* 2003). Total MOI was maintained constant for all treatment groups. Twenty-four hours after infection, cells were treated with CDDP (10 μM) or DMSO for another 24 h and then harvested for further analysis.

3.5 Small interfering RNA (siRNA) transfection

OV2008 and OVCA 432 cells (2.4 x 10^5/well) were seeded in 60 mm dishes. Eighteen hours after plating, 12 μl of transfection reagent was added to 488 μl of RPMI 1640 without serum in 1.5 ml eppendorf tubes. The mixture was vortexed and incubated for 5 min at room temperature. Following incubation, the corresponding concentrations of either p73α siRNA (0-200 nM) for OV2008 and OVCA432 cells or calpain siRNA (0-200 nM) and $p34^{cdc2}$ (0-100 nM) for only OV2008 cells or the scrambled sequences (control) construct was added individually to the tubes. The mixture was then incubated

at room temperature for 15-20 min. During this period, the culture media were removed and the cells were washed once with PBS. About 2,500 µl of complete (10% FBS) media were added and the siRNA mixture was then added in single drops to each well. The cells were returned to the incubator and the media were removed 24 h later and replaced with fresh, complete media for the duration of the culture. Forty-eight hours thereafter, cells were treated with CDDP (10 µM; 24 h) or DMSO and harvested for analysis. Down-regulation of targeted proteins was confirmed by Western blotting.

3.6 Determination of apoptosis

At the end of the culture period, cells were detached from the surface by trypsinization (0.05% trypsin and 0.53 mM EDTA; 37°C, 2 min). Attached and floating cells were pooled, pelleted, and resuspended in neutral-buffered formalin (10%) containing Hoechst 33258 dye (6.25 ng/ml) and after half an hour, cells were ready for apoptotic assessment. They were spotted onto slides and assessed for typical apoptotic nuclear morphology (nuclear shrinkage, condensation, and fragmentation) under a fluorescence microscope with appropriate filter combination (DAPI filter). At least 400 cells/treatment group were counted and selected fields and blinded slides assessed randomly to avoid experimental bias as reported previously (Abedini, Muller *et al.* 2008; Fraser, Bai *et al.* 2008; Abedini, Muller *et al.* 2009).

3.7 Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from cultured OVCA cells was extracted using the RNeasy Mini Kit from Qiagen (Mississauga; ON) according to the manufacturer's instructions and as reported previously (Fraser, Bai *et al.* 2008). Briefly, cell pellets were lysed and homogenized by a buffer containing guanidine isothiocyanate and β-mercaptoehtanol.

The homogenized mixture was spun through QIA shredder columns and an equal volume of 70% ethanol was added to optimize binding of RNA to the silica-gel spin column. The RNA remained bound to the spin column, while the contaminants were removed in several wash steps that followed. RNA was eluted in RNAase-free water and then subjected to DNase I treatment to remove any genomic contamination using the DNA-free kit (Ambion, Austin, TX). Finally, the concentration of the isolated RNA was detected using spectrophotometer. The relative purity was expressed as a ratio of the readings at 260nm and 280nm absorbance.

Aliquots of total RNA were reverse-transcribed (RT) using oligo (dT) primers with Moloney murine leukemia virus (M-MuLV) reverse transcriptase from the Retroscript kit (Ambion, Austin, TX). Briefly, 1 μg of total RNA was incubated with oligo dT primers (2 μg) at 70°C for 5 min to transcribe messenger RNA. A master mix containing 5 x reaction buffers, dNTPs (10 mM), RNase inhibitor (20 IU) was added to the above mixture and incubated at 37°C for 5 min. Finally, 1 μl of RevertAid enzyme reverse transcriptase was added and the first strand cDNA was synthesized at 42°C for 1 h.

Polymerase chain reaction (PCR) was performed using HotStarTaq DNA Polymerase from Qiagen. The PCR amplification reaction started by mixing 0.2 units of the DNA polymerase with 2 μl 10 X PCR buffer, 0.4 μl dNTPs (10 mM each of dATP, dCTP, dGTP and dUTP), 1 μl of cDNA obtained from the above RT reaction and 1 μM of each forward (sense) and reverse (anti-sense) primers in a final volume of 20 μl. PCR primers were from Invitrogen (Burlington, ON) as follows: TAp73α sense: GATTCCAGCATGGACGTCTT, TAp73α antisense: TTCTTCAAGAGCGGGGAGTA,

ΔNp73α sense: AAGCGAAAATGCCAACAAAC, ΔNp73α antisense: GTACGTCCAGGTGGCTGACT and β-actin sense: GGACTTCGAGCAAGAGATGG; β-actin antisense: CACCTTCACCGTTCCAGTTT. PCR conditions following activation (15 min; 95°C) were: denaturation (94°C) for 45 sec, annealing (TAp73α and ΔNp73α: 52°C, β-actin: 54°C) for 45 sec, extension (72°C) for 30 sec, 40 and 25 cycles for p73α and β-actin, respectively. Ten microliters of each PCR product was separated on a 1.5% agarose-ethidium bromide gel and visualized by ultraviolet transillumination using a BioRad GelDoc system.

3.8 Protein extraction and Western blotting

Cells were pelleted and lysed in ice-cold lysis buffer (pH 7.4) containing 50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM NaPPi, 10% glycerol, and 1% Triton X-100. Protease inhibitors Phenylmethylsulfonyl fluoride (PMSF; 1 mM), aprotonin (10 g/l), and Na_3VO_4 (1 mM) were added to the lysis buffer freshly. Cell lysates were sonicated briefly (5 s/cycle) and then incubated on ice for 1 h followed by centrifugation (15 000 *g*; 20 min). The supernatant was taken as whole-cell lysate and stored at -20°C for subsequent analyses. Protein concentration was determined spectrophotometrically using Bio-Rad DC protein assay kit. Equal amounts of proteins (50–80 μg) were loaded and resolved by 8-15% SDS–PAGE and electro-transferred (80 V, 2 h) onto nitrocellulose membranes (Bio-Rad, Canada). Membranes were blocked (room temperature, 1 h) with 5% Blotto (Tris-HCl (10 mM; pH 8.0), NaCl (150 mM), Tween 20 (0.05%, v/v; TBS-Tween 20) containing skim milk (5%; w/v)), then incubated overnight in Blotto at 4°C with the corresponding primary antibodies as described in the reagents section and 1 hour at room temperature with anti-GAPDH, 1 : 20,000). Primary

antibodies were detected with horseradish peroxidase-conjugated goat IgG raised against the corresponding species for one hour at room temperature. Peroxidase activity was visualized with an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) after three washes (15 min/wash) with TBS-Tween 20. Signal intensity was determined densitometrically using Scion Image software, version 4.02 from Scion Corporation (Frederick, MD, USA). Even loading between lanes was determined using Ponceau-S stain and by densitometry of the anti-GAPDH blots.

3.9 Immunoprecipitation (IP)

Cultured cells were treated with CDDP (10μM; 24 h) and then lysed with the standard lysis buffer (1 h, 4°C). After centrifugation, the supernatant was analysed for total protein content (as described above), and 400 μg of total protein were pre-cleared with normal goat IgG and 20 μl G-sepharose beads (GE Healthcare) in a final volume of 500 μl for one hour. All the immunoprecipitation steps were carried out with a gentle rocking at 4°C. The pre-cleared lysate was then incubated with goat anti-p73 (C-17, 2 μg) and 20 μl G-sepharose beads overnight. The agarose beads were pulled down by centrifugation at 3600 x g for 1 minute, and then washed five times with 1 ml lysis buffer with each wash followed by centrifugation (3600 xg; 1 min). After the final wash, 30 μl of 2X SDS loading buffer was added to the beads and boiled for 5 min. The boiled samples were then loaded onto 10% SDS-PAGE gels. Following protein transfer to nitrocellulose, p73α and calpain were detected using Western blot as described above.

3.10 Immunocytochemistry (ICC)

Chemsensitive (OV2008) and chemoresistant (C13*) OVCA cells (25 x 10^3/well) were cultured on 8-well chambers overnight and treated thereafter with either CDDP (10 μM; 12 h) or DMSO. After treatment, cells were washed with serum-free media and fixed with pre-chilled methanol (3 min at -20°C). The fixed cells were blocked with 0.8% (w/v) serum albumin and 1% gelatine in 1X PBS for 20 min at room temperature. Cells were then incubated with rabbit anti-p73α (1:200) and mouse anti-calpain (1:50) primary antibodies overnight (4°C). The cells were washed with 1X PBS (3X; 5mins) and then incubated with donkey anti-rabbit Cy5-(p73α; 1:500) and anti-mouse FITC-conjugated (calpain; 1:200) secondary antibodies. Finally, cells were washed as described earlier and mounted with Vectashield (Vector, Burlingame, CA, USA). Additional wells for each primary antibody were incubated with the corresponding normal IgG as a negative control. Florescence images were acquired using a LSM 510 confocal laser scanning microscope (Zeiss, Germany) with a 63x oil-immersion objective. Channel images were merged using Adobe Photoshop 7.01 (Adobe, Ottawa, Canada).

3.11 Calcium measurement

Chemsensitive (OV2008) and chemoresistant (C13*) OVCA cells (40% confluency) were plated into autoclaved coverslips in "easy grip" culture dishes (Falcon, Franklin Lakes, USA) overnight. Then, cells were loaded with 5 μM of the calcium sensitive Fluo4-AM dye for 30 min at 37°C. Subsequently, the cells were washed twice with serum-free media and in preparation for calcium imaging.

Fluorescent images of OVCA cells were acquired by sequential scanning using an LSM 510 confocal laser-scanning microscope (Zeiss, Germany) with a 40x, 0.8 NA

water-immersion objectives. Sections (1024 x 1024 pixels) were scanned twice to optimize the signal-to-noise ratio. Fields of greater than 6 cells were chosen for measurement, and only one field per coverslip was used. The loaded cells were excited at 488nm and the emission captured at 510 nm using LSM 510 software (Zeiss, Germany).

All experiments were performed at room temperature and the drugs were applied using a constant flow system of approximately 1ml/min as described previously (Yoshida, Monji *et al.* 2006; Splettstoesser, Florea *et al.* 2007). For CDDP experiments, each scan was 2 sec in duration, every 30 sec for a time period of one hour whereas for CPA (100 μM) and caffeine (40 μM), images were taken every 10 sec for a period of 10 min. At least 10 images were acquired prior to bath application of drugs to ensure stable baseline fluorescence. Stacks of images were then loaded into ImageJ (http://rsbweb.nih.gov/ij/) for analysis. A region of interest was drawn around each cell and the fluorescence of each cell was measured independently and imported into a Microsoft Excel spreadsheet. A background adjustment was made by analysing a region of interest located in one corner of the section in which no fluorescent changes were observed, and then subtracting this value from the fluorescence changes observed in the cells. In addition, images taken before the drug application were averaged and subtracted from the fluorescence changes observed during application of the drug. The analyzed data plots are presented as a mean of at least three independent experiments for each data set.

3.12 Statistical analysis

Results are expressed as the mean ± SEM of at least three independent experiments. Depending on the factors tested in each experiment, statistical analysis was carried out by ANOVA using PRISM (Version 3.0 GraphPad, San Diego, CA, USA) and Sigma STAT (Version 3.1, Aspire Software International, Ashburn, VA, USA) softwares, respectively. Differences between multiple experimental factors were determined by the Bonferroni *post hoc* test. Statistical significance was inferred at $^{***}P<0.001$, $^{**}P<0.01$ *and* $^{*}P<0.05$.

CHAPTER 4: RESULTS

4.1 CDDP differentially regulates TAp73α and ΔNp73α mRNA and protein levels in chemosensitive and chemoresistant OVCA cells.

Although the primary aim of the current thesis was to establish the role of TAp73α and ΔNp73α in CDDP-induced apoptosis in ovarian cancer (OVCA) cells, TAp73α and ΔNp73α levels was evaluated first in chemosensitive and chemoresistant OVCA cell lines. Also the effect of CDDP on their mRNA and protein abundance was measured. These findings provided us with concrete conclusions and the right approaches for establishing the role of TAp73α and ΔNp73α in CDDP-induced apoptosis.

To determine the effect of CDDP on TAp73α and ΔNp73α contents and apoptosis, a chemosensitive ovarian cancer (OVCA) cell line (OV2008) and its resistant isogenic counterpart (C13*) were treated with different concentrations of CDDP (0, 2.5, 5 and 10 μM; 24 h) and harvested at different time points (0, 6, 12 and 24 h; 10 μM). TAp73α and ΔNp73α contents and apoptosis were assessed by Western blot and Hoechst stain, respectively. As shown in figure 4.1A, TAp73α was highly expressed in both cell lines while ΔNp73α was only in OV2008 and barely detectable in C13*. CDDP significantly decreased both TAp73α and ΔNp73α contents and induced apoptosis in OV2008 but not C13* in a time- and a concentration-dependent manner.

Next, we examined the effect of CDDP on another pair of OVCA cells; chemosensitive (A2780s) and its resistant variant (p53-mutant A2780cp). Both A2780s and A2780cp cells were treated with CDDP (10 μM) or DMSO (control) and then harvested at different time point (0, 6, 12 and 24 h). TAp73α and ΔNp73α contents and apoptosis were measured as above. CDDP significantly induced apoptosis in a time-

dependent manner in A2780s cells but not A2780cp cells (Figure 4.1B). TAp73α was only detected in A2780cp and CDDP increased its protein content over time. ΔNp73α was not detected at significant levels in either cell line. The lack of TAp73α in A2780s was due to a CpG island methylation at exon 1 (Chen, *et al.* 2000). Treating these cells with different concentrations of 5-Aza Deoxycitidine; a demethylating agent (0, 0.5, 1, 2.5, 5 and 10 μM; 72 h) restored TAp73α content and induced apoptosis (Figure 4.1C).

We observed that TAp73α and ΔNp73α contents increased with the duration of culture in the DMSO-treated group of OV2008 but not in the C13*. This concern was partially addressed in the appendices (Chapter 7; Figure 7.3 and 7.4).

We decided next to assess if the mRNA level of TAp73α and ΔNp73α reflects their protein content in the two pairs of cell lines and whether the decrease in TAp73α and ΔNp73α protein content might be due to decreased mRNA level. In this context, RT-PCR was performed to detect TAp73α and ΔNp73α mRNA abundance. Chemosensitive OVCA cell lines (OV2008 and A2780s) and their resistant counterpart (C13* and A2780cp, respectively) were cultured with CDDP (10 μM; 24 h). Figure 4.2 shows that both TAp73α and ΔNp73α mRNA wereexpressed in all tested cell lines except for TAp73α which was not detected in A2780s due to the CpG methylation as mentioned above. Although ΔNp73α protein content was barely detected in A2780s, C13* and A2780cp, its mRNA level was detected in these cells. CDDP had no effect on TAp73α and ΔNp73α mRNA abundance in the treated cell lines but it increased ΔNp73α mRNA level in A2780cp.

The differential expression of TAp73α and ΔNp73α in OVCA cell lines along with CDDP effect on both their mRNA and protein levels suggested that p73 isoform expression could vary between various cancer cell lines. The several p73 isoforms may also respond differently to drug-induced apoptosis depending on certain criteria (discussed in Chapter 5).

Figure 4.1: The effect of CDDP on TAp73α and ΔNp73α contents and apoptosis in OVCA cells.

CDDP differentially affected TAp73α and ΔNp73α protein contents and apoptosis in chemosensitive and chemoresistant OVCA cells. **A)** Chemosensitive (OV2008) cells and its resistant counterpart (C13*) were treated with different concentrations of CDDP (0, 2.5, 5 and 10 μM; 24 h) and harvested at different time points (0, 6, 12 and 24 h; 10 μM). **B)** Chemosensitive (A2780s) cells and its resistant variant (A2780cp) were treated with different concentrations of CDDP (0, 2.5, 5 and 10 μM; 24 h). **C)** Chemosensitive (A2780s) cells were cultured in different concentrations of 5-Aza Deoxycitidine (0, 0.5, 1, 2.5, 5 and 10 μM; 72 h). TAp73α, ΔNp73α and GAPDH contents and apoptosis were measured by Western blot and Hoechst stain, respectively. Data are represented as the mean ± SEM of three independent experiments. One-way ANOVA was used to assess the effect of CDDP on apoptosis at different concentrations (*** $P < 0.001$).

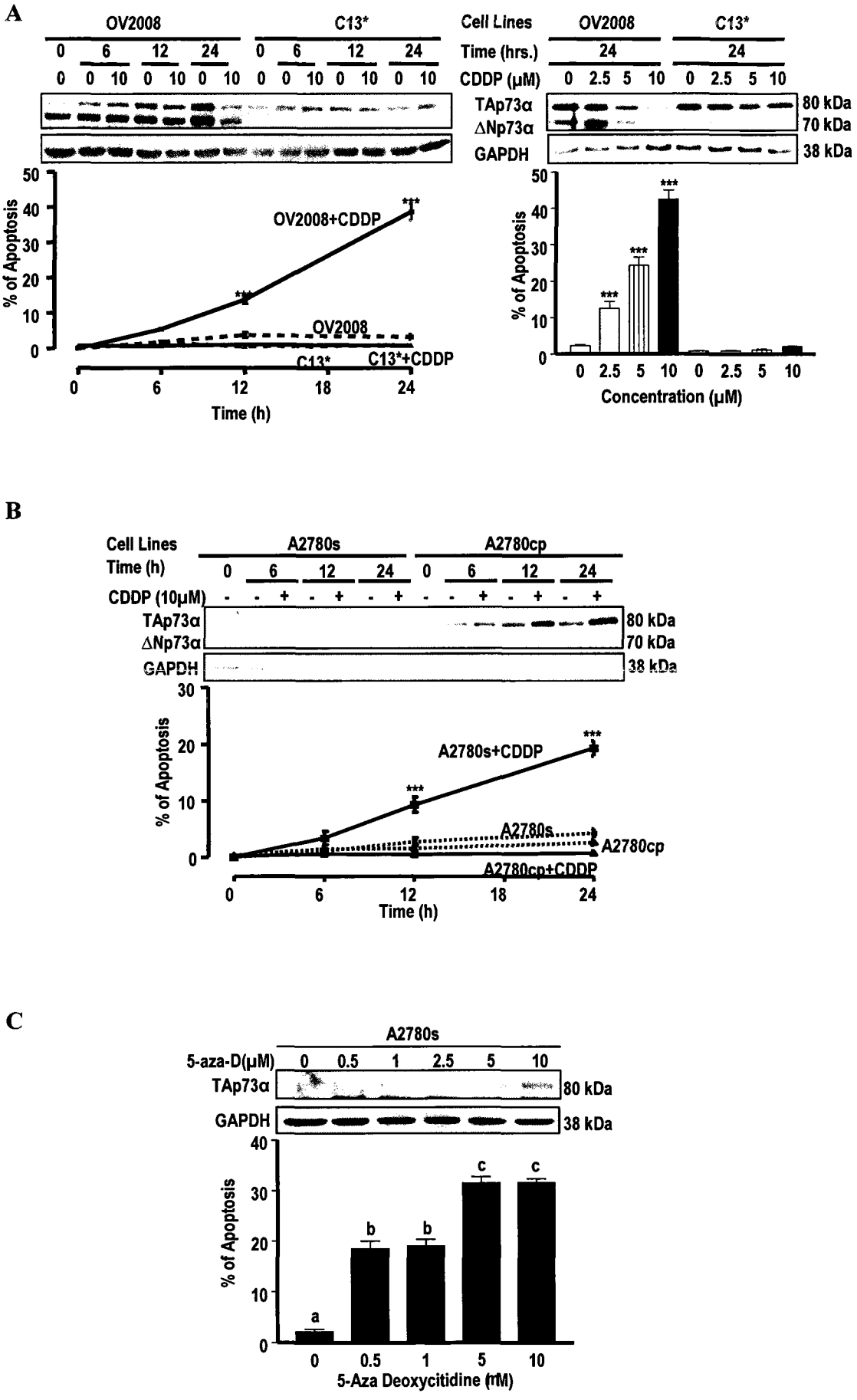

A
OV2008
C13*
Cell Lines
Time (hrs.)
CDDP (μM)
TAp73α
ΔNp73α
GAPDH
80 kDa
70 kDa
38 kDa
% of Apoptosis
OV2008+CDDP
OV2008
C13*
C13*+CDDP
Time (h)
Concentration (μM)
B
A2780s
A2780cp
A2780s+CDDP
A2780cp+CDDP
C
5-aza-D(μM)
5-Aza Deoxycitidine (mM)

Figure 4.1

Figure 4.2: The effect of CDDP on TAp73α and ΔNp73α mRNA level in OVCA cells.

TAp73α and ΔNp73α mRNA abundance and the effects of CDDP varied among OVCA cell lines. Chemosensitive OVCA (OV2008 and A2780s) cells and their resistant counterpart (C13* and A2780cp) were treated with CDDP (10 μM; 24h) or DMSO (control). TAp73α, ΔNp73α and β-actin mRNA levels were measured by RT-PCR. N = 3.

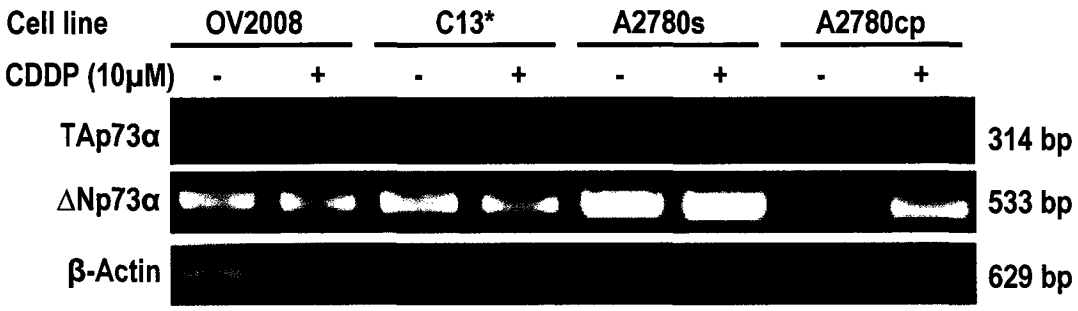

Cell line
OV2008
C13*
A2780s
A2780cp
CDDP (10μM)
- + - + - + - +
TAp73α
ΔNp73α
β-Actin
314 bp
533 bp
629 bp

Figure 4.2

4.2 TAp73α over-expression enhanced CDDP-induced apoptosis in OVCA cells while the effect of ΔNp73α over-expression was variable

Although p73 is an essential mediator in drug-induced apoptosis in some cancer cells (Irwin 2004; Ozaki and Nakagawara 2005; Ramadan, Terrinoni *et al.* 2005), its role in the regulation of chemosensitivity in OVCA is poorly understood. To determine such a role, two pair of chemosensitive OVCA cell line (OV2008 and A2780s) and their resistant isogenic counterparts (C13* and A2780cp, respectively) and an additional CDDP-resistant ovarian cell line (Hey) were transiently transfected with HA-tagged TAp73α, HA-tagged ΔNp73α cDNA (2 μg; 24 h) or empty pcDNA3.1 vector (as control) and then treated with CDDP (5 μM for OV2008; 10 μM for C13*, A2780s, A2780cp and Hey; 24 h).

Effective over-expression of TAp73α and ΔNp73α was confirmed by Western blot (Figure 4.3A, B and C). TAp73α over-expression consistently enhanced CDDP-induced apoptosis compared to the control group in all cell lines tested (OV2008, A2780s, A2780cp and Hey; $P < 0.01$ and C13*; $P < 0.001$). The effect of TAp73α over-expression on CDDP-induced apoptosis was in keeping with the previously published findings (Yoshida, Ozaki et al. 2008; Sang, Ando et al. 2009), indicating that the TA-isoform increases OVCA cell sensitivity to CDDP-induced apoptosis.

The effect of ΔNp73α over-expression was variable. In OV2008 and C13*, as observed with TAp73α, ΔNp73α promotes basal and CDDP-induced apoptosis compared to control (OV2008 ($P < 0.05$); C13* ($P < 0.01$)). In contrast, ΔNp73α failed to sensitise A2780s and Hey cells to CDDP-induced apoptosis but did increase the basal level of apoptosis in A2780cp cells. Our findings of ΔNp73 over-expression is consistent with a

previously published report showing that A2780 clones stably over-expressing ΔNp73α respond to CDDP in a manner similar to their parental cells (Sabatino, Previdi et al. 2007). The enhancement of p73α-induced apoptosis was associated with increased cleavage of PARP, a substrate of caspase-3.

To find out if ΔNp73α-mediated, CDDP-induced apoptosis is also a common phenomenon among the other ΔNp73 isoforms (β and γ), chemoresistant (C13* and A2780cp) OVCA cells were transfected with ΔNp73α, ΔNp73β and ΔNp73γ cDNA (2 μg) or an empty vector (Control). After 24 hours transfection, the cells were treated with CDDP (10 μM) for another 24 hours. The expression of ΔNp73α, ΔNp73β and ΔNp73γ and apoptosis was assessed by Western blot and Hoechst stain, respectively.

All three isoforms showed similar trend in promoting basal apoptosis in both C13* and A2780cp and enhancing CDDP-induced apoptosis only in C13* cells. These findings suggested that ΔNp73α, ΔNp73β and ΔNp73γ function correspondingly in apoptosis in some OVCA cells which, could be regulated by their shared N-terminus and not the diverse C-terminal domain (Chapter 1; Figure 1.4).

Figure 4.3: The effect of TAp73α and ΔNp73α over-expression on CDDP-induced apoptosis in OVCA cell lines.

TAp73α over-expression enhanced CDDP-induced PARP cleavage and apoptosis in OVCA cell lines. The effect of ΔNp73α over-expression was cell-type specific. **A)** OV2008 and C13*, **B)** A2780s and A2780cp and **C)** Hey cells were transfected with either HA-tagged TAp73α cDNA, HA-taged ΔNp73α cDNA (2 μg; 24 h) or an empty vector (control) followed by CDDP (10 μM; 24 h). TAp73α and ΔNp73α over-expression was assessed by Western blot (*Top*), and apoptosis by Hoechst staining (*Bottom*). Effective over-expression of TAp73α and ΔNp73α was confirmed by Western blot using anti-HA antibody. The expression of HA-TAp73α sensitized all the tested cell lines to CDDP-induced apoptosis when compared to the control groups (OV2008, A2780s, A2780cp and Hey; $P < 0.01$ and C13*; $P < 0.001$). The effect of HA-ΔNp73α over-expression was variable. As observed with TAp73α, the over-expression of ΔNp73α promoted basal and CDDP-induced apoptosis in OV2008 and C13* compared to controls [OV2008 ($P < 0.05$); C13* ($P < 0.01$)]. In contrast, ΔNp73α failed to sensitize A2780s and Hey cells to CDDP-induced apoptosis but it did increase the basal level of apoptosis in A2780cp cells. The enhancement of CDDP-induced apoptosis by either isoform was associated with increased cleavage of PARP, a substrate of caspase-3. Figures indicate the mean ± SEM of three independent experiments.

Two way-ANOVA indicates a significant CDDP effect ($P < 0.001$), p73α isoform effect ($P < 0.01$) and significant CDDP-p73 isoform interaction ($P < 0.05$). Bonferroni post hoc test shows significant differences between control and TAp73α [$P < 0.01$ (OV2008, A2780s, A2780cp and Hey); $P < 0.001$ (C13*)] and ΔNp73α ($P < 0.05$) in the presence of CDDP. In the absence of CDDP, basal apoptosis was significantly increased following over-expression of TAp73α [$P < 0.001$ (OV2008 and C13*)] and ΔNp73α [$P < 0.001$ (OV2008, C13* and A2780cp)].

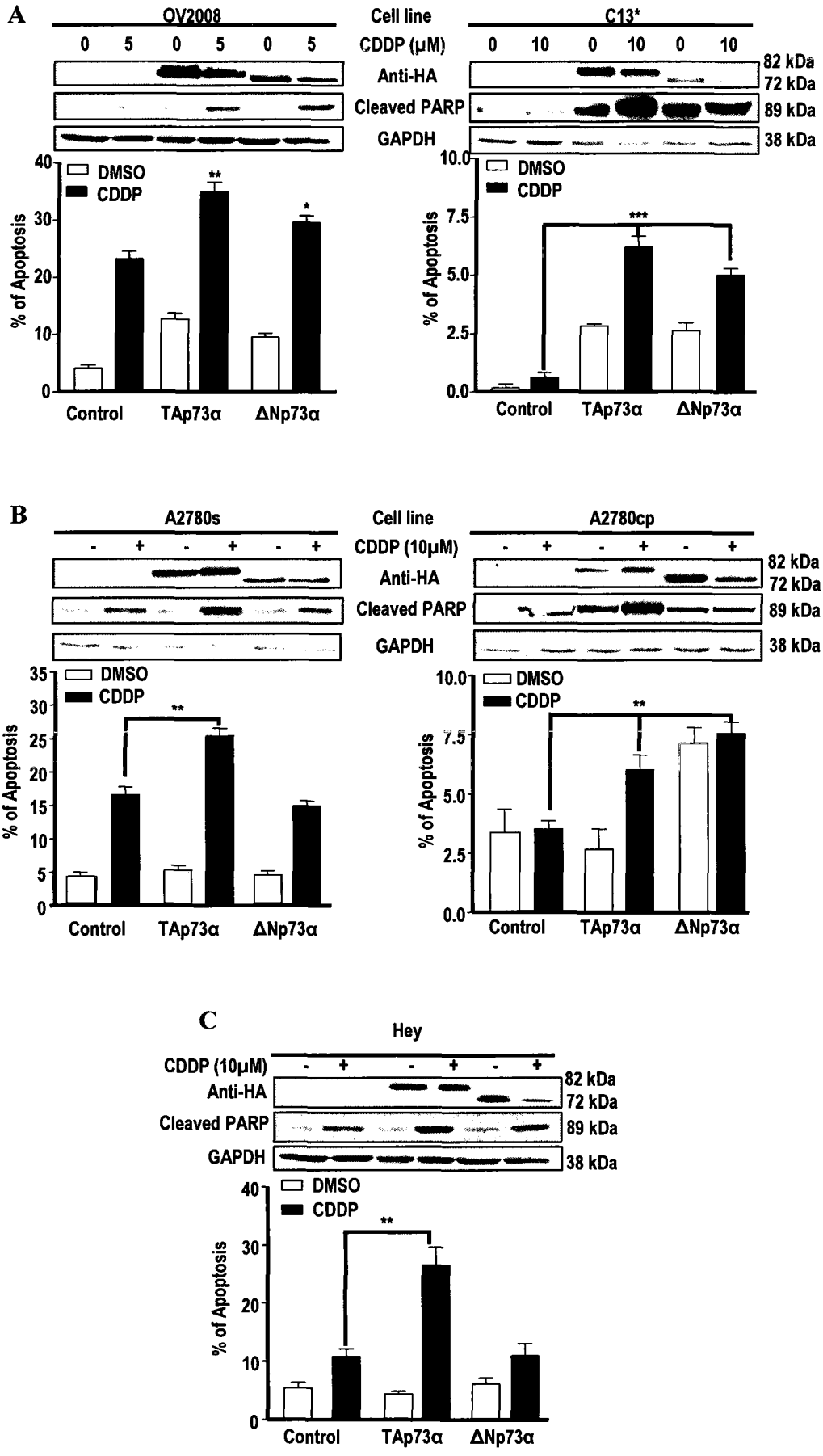

A
OV2008
Cell line
C13*
0 5 0 5 0 5
CDDP (μM)
0 10 0 10 0 10
Anti-HA
Cleaved PARP
GAPDH
82 kDa
72 kDa
89 kDa
38 kDa
DMSO
CDDP
% of Apoptosis
Control
TAp73α
ΔNp73α
B
A2780s
A2780cp
CDDP (10μM)
C
Hey

Figure 4.3

Figure 4.4: The effect of ΔNp73 isoforms (α, β and γ) over-expression on CDDP-induced apoptosis in chemoresistant cells.

Chemoresistant (C13* and 2780cp) OVCA cells were transiently transfected with ΔNp73α, ΔNp73β and ΔNp73γ cDNA (2 μg) or an empty vector (Control) for 24 hours. Then, the cells were treated with CDDP (10 μM) for another 24 hours. Effective over-expression of proteins and GAPDH and apoptosis were assessed by Western Blot and Hoechst Stain, respectively. Data are represented as the mean ± SEM of three independent experiments. Two way-ANOVA indicates a significant CDDP effect ($P < 0.001$), p73α isoform effect ($P < 0.001$) and significant CDDP-p73 isoform interaction ($P < 0.01$). Bonferroni post hoc test shows significant differences between control and ΔNp73α, ΔNp73β and ΔNp73γ [$P < 0.001$ (C13*)] in the presence of CDDP. In the absence of CDDP, basal apoptosis was significantly increased following over-expression of ΔNp73α, ΔNp73β and ΔNp73γ [$P < 0.001$ (C13*) and $P < 0.01$ (A2780cp)].

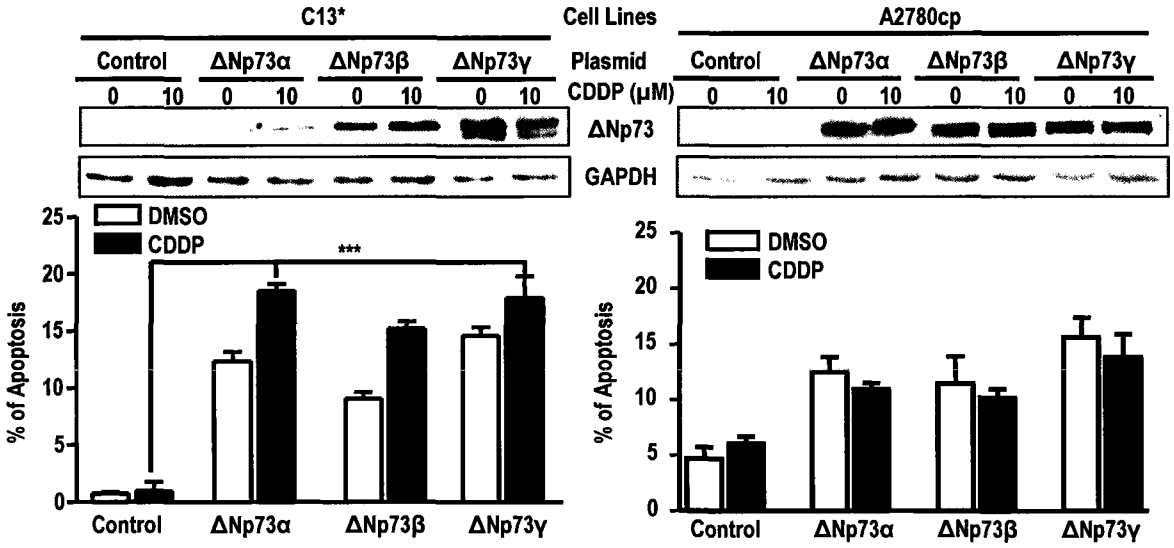

C13*
Cell Lines
A2780cp
Control
ΔNp73α
ΔNp73β
ΔNp73γ
Plasmid
CDDP (μM)
0
10
ΔNp73
GAPDH
DMSO
CDDP
***
% of Apoptosis
25
20
15
10
5
0
Control
ΔNp73α
ΔNp73β
ΔNp73γ

Figure 4.4

4.3 P73α is involved in CDDP-induced apoptosis in chemosensitive OVCA cells.

To further examine the role of TAp73α and ΔNp73α in the regulation of CDDP sensitivity, chemosensitive (OV2008) cells were transfected with p73α siRNA targeting both isoforms (0, 50, 100 and 200 nM; 48 h) prior to treatment with CDDP (10 μM; 24 h). TAp73α and ΔNp73α contents and apoptosis were assessed as above.

In the controls (untransfected cells), CDDP decreased TAp73α and ΔNp73α content and induced apoptosis in OV2008 cells (Figure 4.5A). P73α siRNA markedly decreased TAp73α and ΔNp73α contents in a concentration-dependent manner and significantly attenuated CDDP-induced apoptosis compared to the control-group ($P<0.01$). Although p73α protein was almost completely down-regulated by siRNA, CDDP-induced apoptosis was not totally suppressed. The lack of complete apoptosis suppression could be attributed to the other p73 isoforms such as TAp73β, which have also been shown to induce apoptosis upon DNA damage (Das, Nama *et al.* 2005). In addition to that, p73-independent pathways such as p53-mediated apoptosis may contribute to the remaining percentage of CDDP-induced apoptosis in OVCA cells.

The high level of homology in the DNA binding domain of both p53 and TAp73 allows the later to bind and transcriptionally activate p53-responsive genes to induce apoptosis (Agami, Blandino *et al.* 1999; Di Como, Gaiddon *et al.* 1999; Fang, Lee *et al.* 1999). It has been shown that TAp73-induced apoptosis is mediated by PUMA induction, resulting in Bax mitochondrial translocation and cytochrome c release (Melino, Bernassola *et al.* 2004). NOXA expression is also up-regulated by p73 activation upon ionizing irradiation and UV treatment in melanocyte and keratinocyte carcinomas (Kulesz-Martin, Lagowski *et al.* 2005). Whether PUMA or NOXA are down-stream

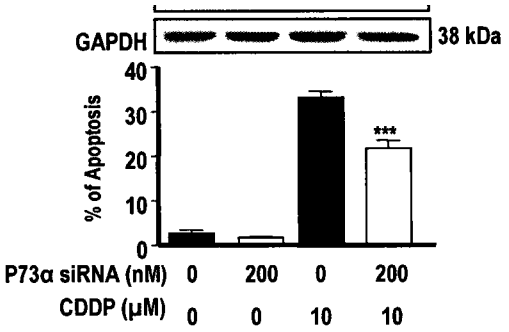
effectors of p73α-mediated apoptosis upon CDDP treatment has not been examined yet in OVCA cells. In order to investigate PUMA- and NOXA-mediated function in CDDP-induced apoptosis, cell lysate obtained form chemosensitive OVCA cells (OV2008) transfected with either p73α siRNA (200 nM) or controlled siRNA were ran in 15% SDS-PAGE gel. P53-responsive gene products PUMA and NOXA were detected using rabbit anti-PUMA and mouse anti-NOXA antibody respectively.

Figure 4.5B illustrated a significant increase in PUMA and NOXA contents and apoptosis induction upon CDDP treatment compared to the DMSO-treated group. P73α siRNA markedly decreased TAp73α and ΔNp73α contents and significantly attenuated these responses induced by CDDP (DMSO *v.s.* CDDP; $P < 0.001$). P73 siRNA had no effect on p53 content, suggesting that the changes in PUMA and NOXA content were specific to p73α and not a secondary effect to a decrease in p53 content. These results suggested that PUMA and NOXA are down-stream effectors of CDDP-induced p73α-mediated apoptosis in OV2008 cells.

To provide further evidence that the endogenous p73α is required for CDDP-induced apoptosis in OVCA cells, another chemosensitive (p53-mutant; OVCA432) cells were transfected with p73α siRNA (200 nM) as described above. While ΔNp73α was not expressed in these cells, CDDP significantly increased TAp73α content and induced apoptosis compared to the non-treated group (Figure 4.5C; $P < 0.05$). TAp73α down-regulation by p73α siRNA attenuated CDDP-induced apoptosis in OVCA432 cells. The lack of ΔNp73α expression as well as CDDP-induced TAp73α increase supports the notion that p73 isoforms are differentially expressed and differentially regulated by CDDP in various OVCA cells.

Figure 4.5: The effect of p73α down-regulation on CDDP-induced apoptosis in chemosensitive cells.

P73α down-regulation by p73α siRNA attenuated CDDP-induced apoptosis in chemosensitive cells (OV2008 and OVCA 432). It also decreased PUMA and NOXA content in CDDP-treated group in OV2008 cells. **A** and **B)** OV2008 and **C)** OVCA432 cells were transfected with p73α siRNA (0-200 nM; 48 h) and treated thereafter with CDDP (10 μM; 24 h). TAp73α, ΔNp73α, PUMA and NOXA contents (*Top*) and apoptosis (*Bottom*) were determined by Western blot and Hoechst stain, respectively. P73α siRNA markedly down-regulated TAp73α and ΔNp73α contents and had no effect on p53 content. TAp73α and ΔNp73α down-regulation significantly attenuated CDDP-induced apoptosis compared to siRNA controlled-group treated with CDDP. Figures are presented as the mean ± SEM of three independent experiments. Two way-ANOVA indicates a significant CDDP effect ($P < 0.001$), p73α siRNA effect ($P < 0.05$) and significant CDDP-p73 siRNA interaction ($P < 0.05$). Bonferroni post hoc test shows significant differences between control siRNA and p73α siRNA [$P < 0.001$ (OV2008); $P < 0.05$ (OVCA432)] in the presence of CDDP.


GAPDH
38 kDa
40
30
20
10
0
% of Apoptosis
***
P73α siRNA (nM) 0 200 0 200
CDDP (μM) 0 0 10 10

C

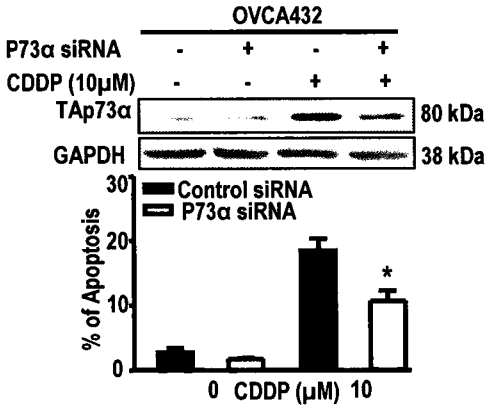
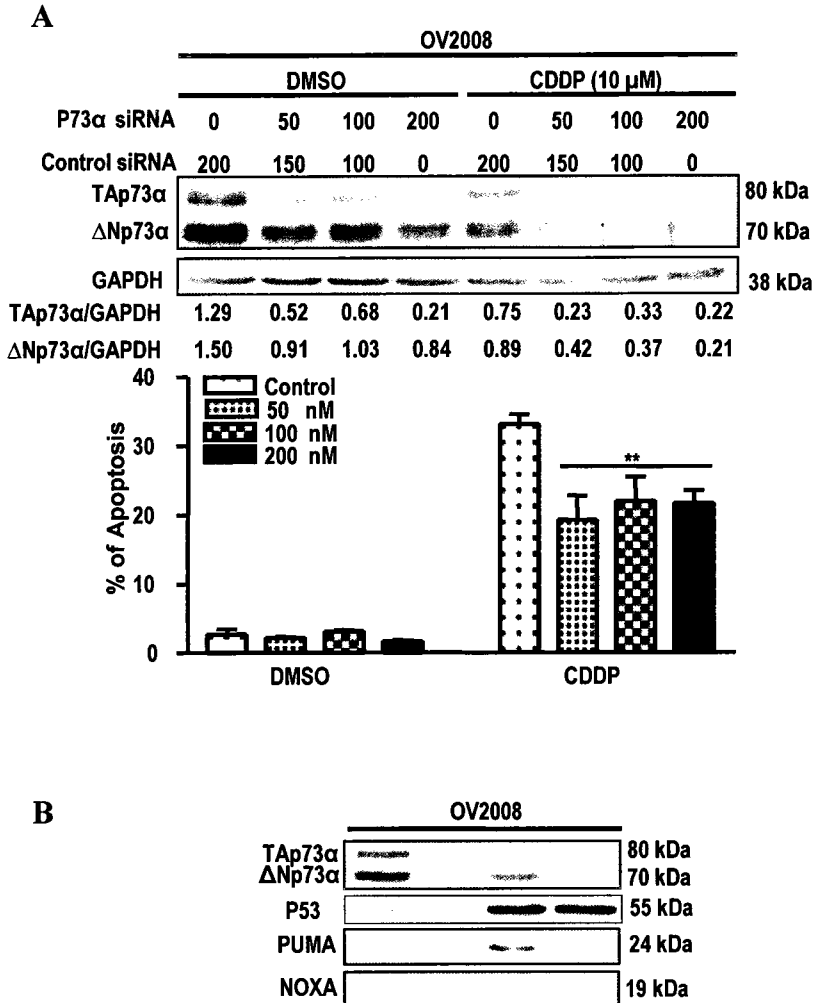

OVCA432
P73α siRNA - + - +
CDDP (10μM) - - + +
TAp73α
80 kDa
GAPDH
38 kDa
Control siRNA
P73α siRNA
30
20
10
0
% of Apoptosis
*
0 CDDP (μM) 10

Figure 4.5


A
OV2008
DMSO
CDDP (10 μM)
P73α siRNA 0 50 100 200 0 50 100 200
Control siRNA 200 150 100 0 200 150 100 0
TAp73α
ΔNp73α
80 kDa
70 kDa
GAPDH
38 kDa
TAp73α/GAPDH 1.29 0.52 0.68 0.21 0.75 0.23 0.33 0.22
ΔNp73α/GAPDH 1.50 0.91 1.03 0.84 0.89 0.42 0.37 0.21
% of Apoptosis
40
30
20
10
0
Control
50 nM
100 nM
200 nM
**
DMSO
CDDP
B
OV2008
TAp73α
ΔNp73α
80 kDa
70 kDa
P53
55 kDa
PUMA
24 kDa
NOXA
19 kDa

4.4 CDDP-induced p73α down-regulation is not affected by proteasome inhibition or cell-death blockage in chemosensitive cells

Chemosensitive cells (OV2008) and its resistant counterpart (C13*) were used for the subsequent studies. Chemosensitive (A2780s) and chemoresistant (A2780cp) cells were excluded due to the lack of TAp73α and ΔNp73α expression as described in figure 4.1.

Once the role of TAp73α and ΔNp73α in OV2008 and C13* cells were tested, we next examined the mechanisms by which CDDP regulates TAp73α and ΔNp73α content in chemosensitive cells. We showed that CDDP decreased TAp73α and ΔNp73α content in OV2008 cell but not C13* cells. Since OV2008 cells are CDDP-responsive and undergo apoptosis when challenged with CDDP, it is possible that the decreased TAp73α and ΔNp73α content induced by CDDP is a consequence of cell death. To rule out this possibility, OV2008 cells were infected with adenovirus carrying Xiap sense cDNA or LacZ (MOI = 5; 24 h) followed by CDDP (10 μM; 24 h). Our laboratory has previously demonstrated that Xiap, an endogenous anti-apoptotic protein, inhibits CDDP-induced apoptosis through suppressing caspase-3 activity in OVCA cells (Fraser, Leung *et al.* 2003). Xiap over-expression, p73α isoforms contents and cleaved-PARP and apoptosis were assessed by Western blotting and Hoechst stain, respectively.

As shown in figure 4.6, immuno-blotting demonstrated a significant increase in Xiap protein level in both control and CDDP-treated cells infected with Xiap. CDDP decreased Xiap and TAp73α and ΔNp73α contents, increased cleaved PARP and induced apoptosis in both LacZ and Xiap expressing groups. Although the over-expression of Xiap significantly attenuated PARP cleavage and CDDP-induced apoptosis (LacZ *v.s.*

Xiap; $P < 0.001$), it had no effect on CDDP-induced TAp73α and ΔNp73α down-regulation in OV2008 cells, suggesting that this down-regulation is specific and not a consequence of cell death. In addition, the CDDP-induced Xiap decrease did not prevent the attenuation of CDDP-induced apoptosis in Xiap-infected cells treated with CDDP, suggesting that minimal quantity of Xiap might be enough to exert its anti-apoptotic function in these cells.

The p73 protein degradation can be regulated by mechanisms in part through the ubiquitin-proteasome system (discussed in Chapter 1). To examine whether the proteasome pathway is involved in CDDP-induced TAp73α and ΔNp73α down-regulation, OV2008 cells were pre-treated for 30 minutes with Epoximicin (10 or 20 nM) or Lactacystine (4 or 8 μM) and then treated with CDDP (10 μM; 24 h). While these inhibitors significantly attenuated CDDP-induced apoptosis and increased p53 content (positive control) in OV2008 cells, they failed to attenuate CDDP-induced TAp73α and ΔNp73α down-regulation (Figure 4.7). TAp73α and ΔNp73α content, however, decreased at higher concentrations of the inhibitors, presumably due to the cytotoxicity. Together, these results demonstrated that the proteasome pathway is not likely to be involved in CDDP-induced TAp73α and ΔNp73α down-regulation in the OV2008 chemosensitive cells.

Figure 4.6: The effect of Xiap over-expression on TAp73α and ΔNp73α content and apoptosis in chemosensitive cells.

CDDP-induced p73α down-regulation is not a consequence of cell-death. Chemosensitive (OV2008) cells was infected with adenovirus carrying Xiap sense cDNA or LacZ (5 MOI; 48 h) and then treated with CDDP (10 μM; 24 h). Xiap over-expression, TAp73α, ΔNp73α and GAPDH contents and cleaved PARP and apoptosis were assessed by Western blot and Hoechst stain, respectively. Data are represented as the mean ± SEM of three independent experiments. Two way-ANOVA indicates a significant CDDP effect ($P < 0.001$), Xiap effect ($P < 0.001$) and significant CDDP-Xiap interaction ($P < 0.01$). Bonferroni post hoc test shows significant differences between LacZ (control) and Xiap infection ($P < 0.001$) in the presence of CDDP.

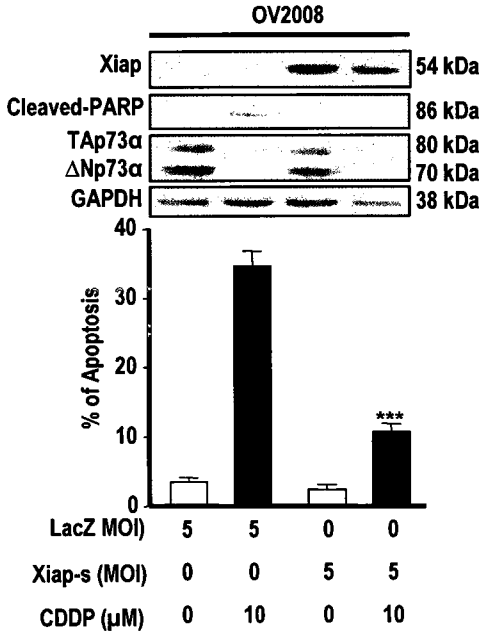

OV2008
Xiap
Cleaved-PARP
TAp73α
ΔNp73α
GAPDH
54 kDa
86 kDa
80 kDa
70 kDa
38 kDa
% of Apoptosis
40
30
20
10
0
***
LacZ MOI) 5 5 0 0
Xiap-s (MOI) 0 0 5 5
CDDP (μM) 0 10 0 10

Figure 4.6

Figure 4.7: The effect of the proteasome inhibitors on TAp73α and ΔNp73α content and apoptosis in chemosensitive cells.

CDDP-induced, TAp73α and ΔNp73α down-regulation was not affected by proteasome inhibition in chemosensitive cells (OV2008). OV2008 cells were pre-treated for 30 minutes with different concentrations of Epoximicin (10 or 20 nM) or Lactacystine (4 or 8 μM) followed by CDDP treatment (10 μM; 24 h). TAp73α, ΔNp73α, p53 and GAPDH contents and apoptosis were assessed by Western blot and Hoechst stain, respectively. Data are represented as the mean ± SEM of three independent experiments. Three way-ANOVA indicates a significant CDDP effect ($P < 0.001$), Epoximicin effect ($P < 0.01$) and Lactacystine effect ($P < 0.01$) and significant CDDP-Epoximicin interaction ($P < 0.05$) and CDDP-Lactacystine interaction ($P < 0.05$). Bonferroni post hoc test shows significant differences between control and Epoximicin ($P < 0.01$) and Lactacystine ($P < 0.05$) in the presence of CDDP.

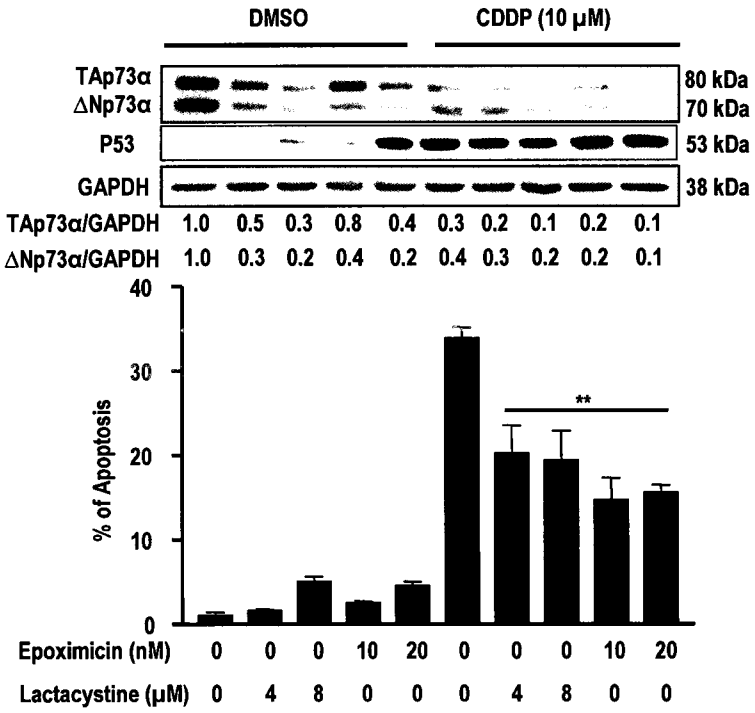

DMSO
CDDP (10 μM)
TAp73α
ΔNp73α
P53
GAPDH
80 kDa
70 kDa
53 kDa
38 kDa
TAp73α/GAPDH 1.0 0.5 0.3 0.8 0.4 0.3 0.2 0.1 0.2 0.1
ΔNp73α/GAPDH 1.0 0.3 0.2 0.4 0.2 0.4 0.3 0.2 0.2 0.1
% of Apoptosis
40
30
20
10
0
**
Epoximicin (nM) 0 0 0 10 20 0 0 0 10 20
Lactacystine (μM) 0 4 8 0 0 0 4 8 0 0

Figure 4.7

4.5 CDDP-induced p73α down-regulation is mediated by a calpain-dependent pathway.

Calpain regulates the steady-state level of various p73 isoforms (discussed in Chapter 1). In order to test the participation of calpain in CDDP-induced TAp73α and ΔNp73α down-regulation, the OV2008 cells were pre-treated for 4 hours with different concentrations of calpeptin (0, 6.25, 12.5, 25 and 50 μM) followed by CDDP treatment (10 μM; 24 h). As shown in figure 4.8A, CDDP activated calpain (as indicated by the presence of cleaved α-fodrin), and this was inhibited by calpeptin, which also attenuated CDDP-induced apoptosis in a concentration-dependent manner. Calpain inhibition restored TAp73α and ΔNp73α contents, a maximal response noted at 25 μM calpeptin.

To further confirm the effect of calpain inhibition on TAp73α and ΔNp73α contents, OV2008 cells were transfected with either calpain siRNA (targeting the small regulatory subunit of calpain; 100 and 200 nM; 48 h) or scrambled sequence siRNA (control) and treated with CDDP (10 μM; 24 h). Calpain siRNA successfully decreased calpain content and activation (illustrated by cleaved α-fodrin). Calpain down-regulation significantly attenuated CDDP-induced TAp73α and ΔNp73α down-regulation and apoptosis in the CDDP-treated cells (Figure 4.8B). The CDDP-induced apoptosis was not completely reduced to the control (DMSO) levels when p73α contents were restored due to the presence of other calpain-mediated p73α-independent apoptosis.

To validate the processing of TAp73α and ΔNp73α by calpain, OV2008 cell lysates were incubated with recombinant calpain 1 followed by Western blot analysis. The Ca^{2+}-activated calpain 1 induced α-fodrin cleavage and TAp73α and ΔNp73α processing; however, these events were prevented in the presence of EGTA or when calpain was inactivated by boiling (Figure 2E). The Calpain-mediated α-fodrin cleavage and TAp73α and ΔNp73α processing were consistent with the different concentration of Ca^{2+}, suggesting that calpain is activated by Ca^{2+}. Unfortunately, the antibody used to detect TAp73α and ΔNp73α content failed to recognize their cleaved products induced by calpain 1. Collectively, our studies provided strong evidence for an involvement of calpain in the regulation of TAp73α and ΔNp73α protein level both alone or in the presence of CDDP.

Figure 4.8: The effect of calpain inhibition on TAp73α and ΔNp73α content and apoptosis in chemosensitive cells.

CDDP-induced TAp73α and ΔNp73α down-regulation is calpain-dependent. Calpain inhibition by either calpeptin **A)** or specific calpain siRNA **B)** decreased cleaved α-fodrin, restored TAp73α and ΔNp73α content and attenuated CDDP-induced apoptosis. OV2008 cells were pre-treated for 4 h with different concentrations of calpeptin (0, 6.25, 12.5, 25 and 50 μM) or transfected with calpain siRNA (0 - 200 nM; 48 h). **C)** OV2008 cell lysates were incubated with recombinant calpain 1 for one hour at 30°C, where calpain activity was inhibited by boiling, EGTA and absence of Ca^{2+}. **E)** OV2008 cells lysate was incubated as above and calpain was activated by increasing concentration of Ca^{2+}. Calpain down-regulation, TAp73α and ΔNp73α contents, cleaved α-fodrin and apoptosis were assessed by Western blot and Hoechst stain. Data are represented as the mean ± SEM of three independent experiments. Two way-ANOVA indicates a significant CDDP effect ($P < 0.001$), A) calpeptin effect ($P < 0.05$), B) calpain siRNA effect ($P < 0.05$) and significant CDDP-calpeptin interaction ($P < 0.01$) and CDDP-calpain siRNA interaction ($P < 0.05$). Bonferroni post hoc test shows significant differences between control and calpeptin [$P < 0.05$] in CDDP-treated groups. It also shows significant differences between control siRNA and calpain siRNA [$P < 0.01$] in the presence of CDDP.

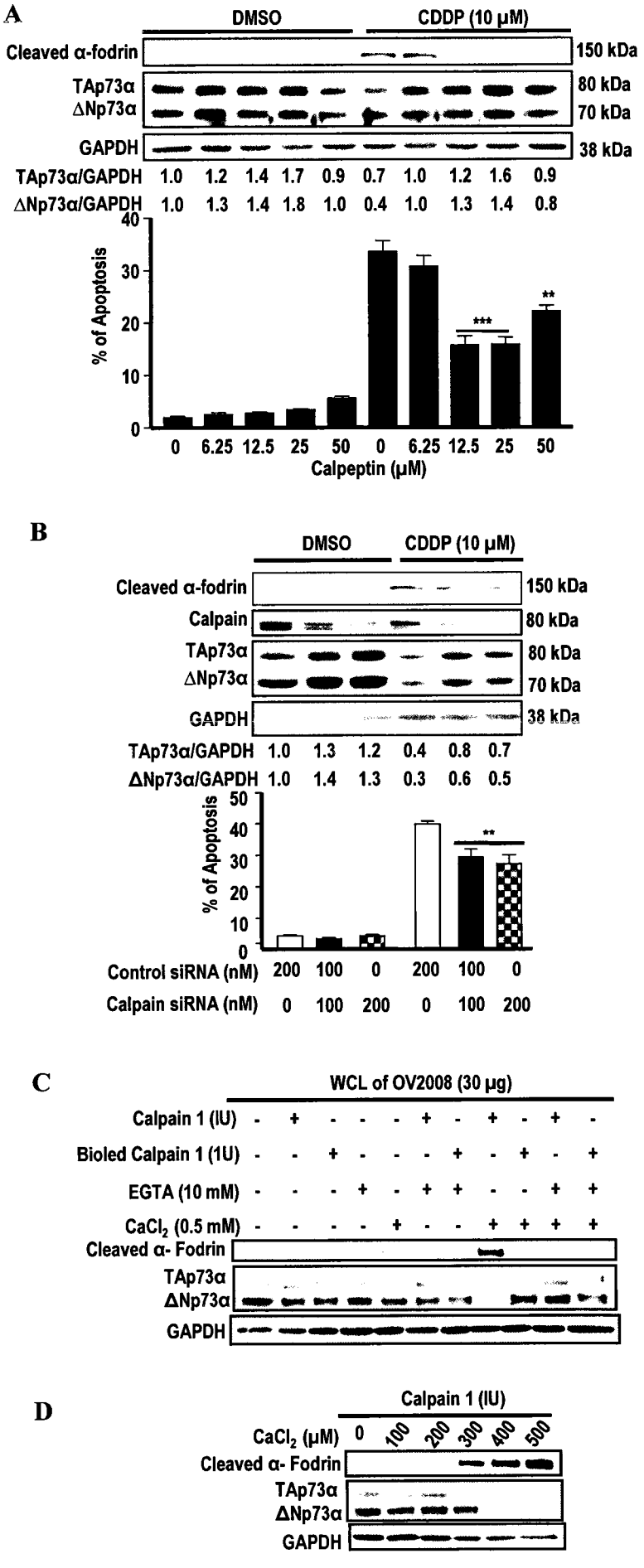

A
DMSO
CDDP (10 μM)
Cleaved α-fodrin
150 kDa
TAp73α
ΔNp73α
80 kDa
70 kDa
GAPDH
38 kDa
TAp73α/GAPDH 1.0 1.2 1.4 1.7 0.9 0.7 1.0 1.2 1.6 0.9
ΔNp73α/GAPDH 1.0 1.3 1.4 1.8 1.0 0.4 1.0 1.3 1.4 0.8
% of Apoptosis
0 10 20 30 40
***
**
0 6.25 12.5 25 50 0 6.25 12.5 25 50
Calpeptin (μM)
B
DMSO
CDDP (10 μM)
Cleaved α-fodrin
150 kDa
Calpain
80 kDa
TAp73α
80 kDa
ΔNp73α
70 kDa
GAPDH
38 kDa
TAp73α/GAPDH 1.0 1.3 1.2 0.4 0.8 0.7
ΔNp73α/GAPDH 1.0 1.4 1.3 0.3 0.6 0.5
% of Apoptosis
0 10 20 30 40 50
**
Control siRNA (nM) 200 100 0 200 100 0
Calpain siRNA (nM) 0 100 200 0 100 200
C
WCL of OV2008 (30 μg)
Calpain 1 (IU) - + - - - + - + - + -
Bioled Calpain 1 (1U) - - + - - - + - + - +
EGTA (10 mM) - - - + - + + - - + +
CaCl2 (0.5 mM) - - - - + - - + + + +
Cleaved α- Fodrin
TAp73α
ΔNp73α
GAPDH
D
Calpain 1 (IU)
CaCl2 (μM) 0 100 200 300 400 500
Cleaved α- Fodrin
TAp73α
ΔNp73α
GAPDH

Figure 4.8

4.6 CDDP enhanced p73α and calpain interaction and co-localization in chemosensitive but not chemoresistant cells.

Given that CDDP-induced TAp73α and ΔNp73α down-regulation is regulated by calpain and only occurred in chemosensitive OV2008 cells, it would be useful to determine if CDDP has a differential effect on calpain activity in both chemosensitive (OV2008) and chemoresistant (C13*) cells. In order to assess calpain activation, both cells were treated with different concentrations of CDDP (0, 2.5, 5 and 10 μM; 24 h) and then harvested at different time points (0, 6, 12, and 24 h; 10 μM). Calpain activation was determined by α-fodrin cleavage using Western blot. As shown in figure 4.9, CDDP induced α-fodrin cleavage, detected at 12 hours post treatment, in OV2008 but not C13* cells in a concentration-dependent manner, supporting the notion that the absence of CDDP-induced p73α processing in C13* might be due to the lack of calpain activation.

Since calpain cleaves p73 at two putative sites (discussed in Chapter 1), we speculated that calpain and p73α isoforms may interact and such interaction could be influenced by CDDP. To test this hypothesis, OV2008 and C13* were treated with CDDP (10 μM; 24 h) and the interaction between calpain and p73α isoforms were examined by co-immunoprecipitation (described in Chapter 3).

As demonstrated in figure 4.10A, both p73α isoforms and calpain were successfully co-precipitated with an anti-p73 antibody, a response enhanced in OV2008 (0.25 *v.s* 0.7) but not C13* (0.5 *v.s* 0.5) cells following CDDP treatment. Taken together, these findings support the hypothesis that CDDP enhances p73α-calpain interaction and such interaction is associated with CDDP-induced, calpain-mediated p73α down-regulation/cleavage.

Although it has been shown that calpain cleaved p73 at both the N- and C-terminus, the consensus site of calpain cleavage in p73 sequence is unknown. Due to the wide variations between the p73 isoforms at both termini where calpain cleavage site is present, it was currently difficult to construct a mutant p73 that lack these sites in order to study the functional consequences of such cleavage (discussed in Chapter 5).

Immunolocalization studies (described in Chapter 3) on p73α in OV2008 treated with DMSO (control) showed immunoreactivity for p73α localized predominantly in the nucleus as well as in the perinuclear region (Figure 4.10B). CDDP treatment (10 μM; 24 h) resulted in a decrease in nuclear p73α immunoreactivity and increased localization in the cytoplasm. In contrast, p73α immunoactivity was low or not detectable in the nucleus of C13* and was not influence by the presence of CDDP. Calpain immunoreactivity in OV2008 cells was only found at the cytoplasm and was not affected by CDDP (Figure 3C). Overlay of p73α and calpain immunoreactivities illustrated that CDDP treatment of OV2008 cells results in co-localization of immunosignals in clusters with increased intensity in the cytoplasm. However, C13* cells exhibited a similar immunoreactive pattern in the absence and presence of CDDP, in which the co-localization of p73 and calpain was evident only in the cytoplasm, raising the possibility that differential co-localization of both proteins in the two cell lines could be an essential pre-requisite for the subsequent regulation of p73α by calpain.

Figure 4.9: The effect of CDDP on calpain activation in OVCA cells.

CDDP induced calpain activation (illustrated by cleaved α-fodrin) in OV2008 but not C13*. Both cells were treated with different concentration of CDDP (0, 2.5, 5 and 10 μM; 24 h) and harvested at different time points (0, 6, 12 and 24 h; 10 μM). Calpain activation was evident by the presence of cleaved α-fodrin on Western blotting.

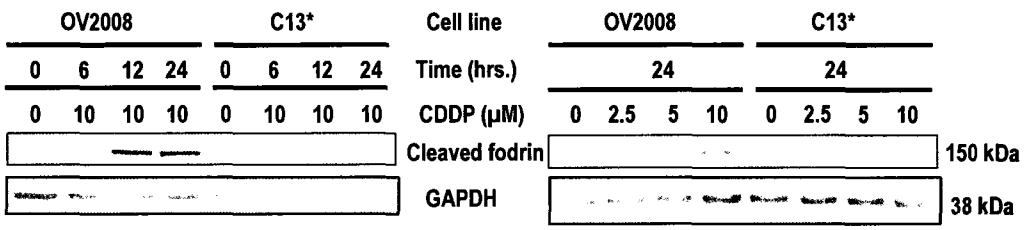
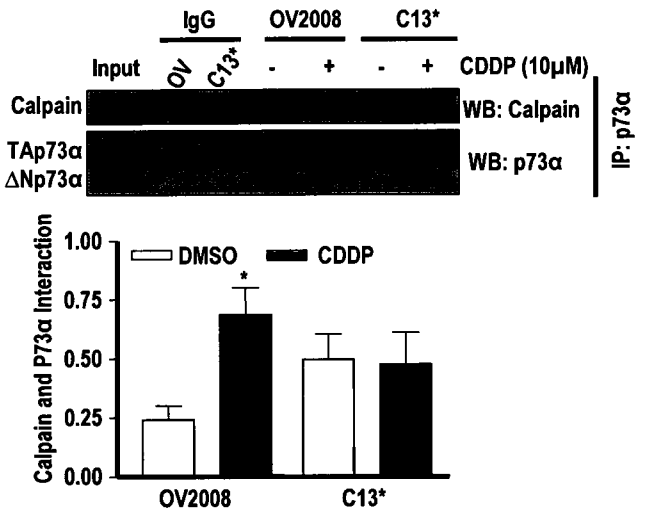

OV2008
C13*
Cell line
OV2008
C13*
0 6 12 24 0 6 12 24
Time (hrs.)
24
24
0 10 10 10 0 10 10 10
CDDP (μM)
0 2.5 5 10 0 2.5 5 10
Cleaved fodrin
150 kDa
GAPDH
38 kDa

Figure 4.9

Figure 4.10: The effect of CDDP on calpain-p73α interaction and co-localization in OVCA cells.

CDDP enhanced calpain and p73α interaction and co-localization in OV2008 but not C13* cells. **A)** CDDP enhanced p73α and calpain interaction in OV2008 but not C13* cells. P73α was immunoprecipitated and p73α and calpain binding was detected by Western blot. Data are represented as the mean ± SEM of three independent experiments. Two way-ANOVA indicates a significant CDDP effect ($P < 0.05$), cell line effect ($P < 0.05$). Bonferroni post hoc test shows significant differences between control (DMSO) and CDDP ($P < 0.05$) in OV2008. **B)** CDDP enhanced the cytoplasmic co-localization of p73α and calpain in OV2008 but not C13* cells. Both OV2008 and C13* cells were treated CDDP (10 μM; 12 h). P73α and calpain co-localization was detected by immunocytochemistry. The images were a representative of three independent experiments.

A



B

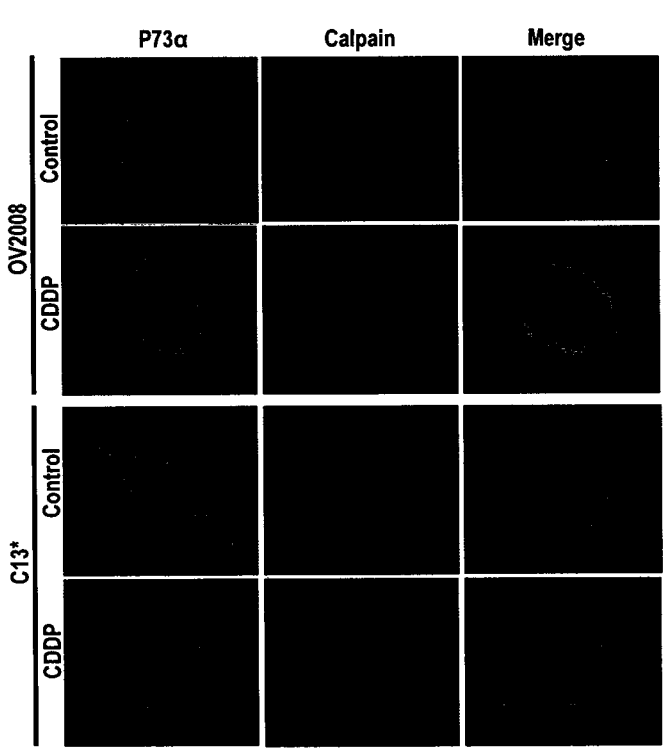


Figure 4.10

4.7 CDDP increased intracellular calcium concentration $[Ca^{2+}]_i$ in chemosensitive but not chemoresistant cells.

Since calpain is a Ca^{2+}-dependent protease, we next examined whether CDDP influences the Ca^{2+} homeostasis in chemosensitive and chemoresistant OVCA cells. OV2008 and C13* cells were subjected to calcium imaging as described in chapter 3. Briefly, the cells were cultured on coverslips and loaded with 5 μM of Fluo-4/AM dye for 30 minutes at 37°C. Fluorescent images were acquired by sequential scanning using an LSM 510 confocal laser-scanning microscope (Zeiss, Germany), representing the changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) caused by CDDP (10 μM) that was applied to the alive cells by a constant flow system of approximately 1ml/min.

Representative images of OV2008 cells showed a marked increase in the green fluorescence of the dye while there was no difference in C13* (Figure 4.11). CDDP caused a significant increase in the $[Ca^{2+}]_i$ compared to the DMSO-groups in OV2008 but not in C13* cells. The increases in $[Ca^{2+}]_i$ were evident around 15 minutes following CDDP addition to the cultures and continued to increase up to 50 minutes, when the experiment was terminated. The differential influence of CDDP on $[Ca^{2+}]_i$, calpain activation and p73α processing in the chemosensitive and chemoresistant cells suggest that these pathways could regulate OVCA cell sensitivity to CDDP in some OVCA cells such as OV2008 and C13*.

Figure 4.11: The effect of CDDP on the $[Ca^{2+}]_i$ in OVCA cells.

CDDP induced $[Ca^{2+}]_i$ increase in chemosensitive (OV2008) but not the chemoresistant (C13*) cells. The effect of CDDP on the $[Ca^{2+}]_i$ in OV2008 and C13* cells was assessed by confocal microscopy. Data are represented as the mean ± SEM of four independent experiments. Two way-ANOVA indicates a significant CDDP effect ($P < 0.001$) and cell line effect ($P < 0.05$) on $[Ca^{2+}]_i$ and significant CDDP-cell line interaction ($P < 0.01$). Bonferroni post hoc test shows significant differences between DMSO and CDDP [$P < 0.001$] in OV2008 but not C13*.

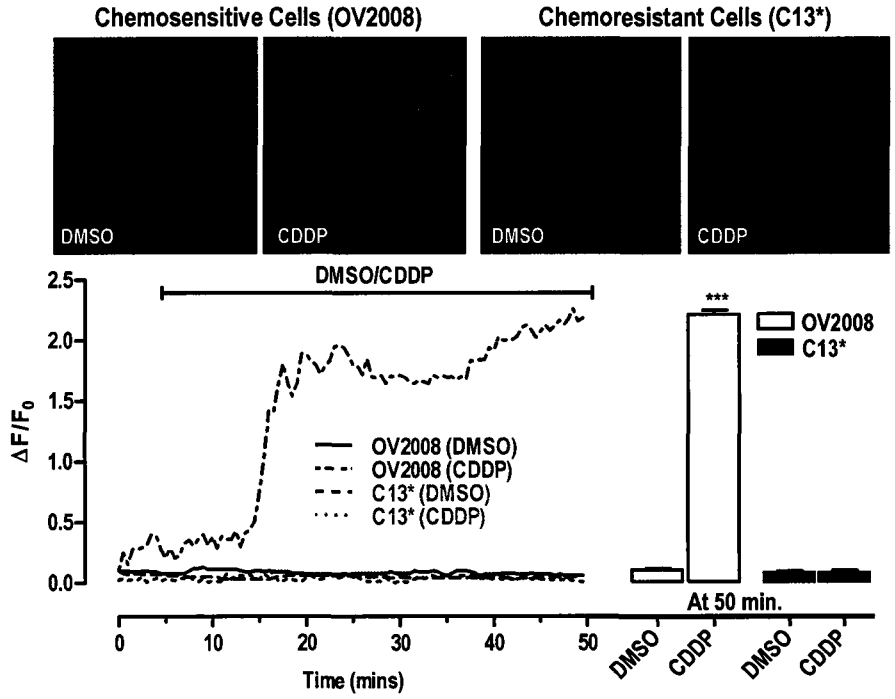

Chemosensitive Cells (OV2008)
Chemoresistant Cells (C13*)
DMSO
CDDP
DMSO
CDDP
DMSO/CDDP
2.5
2.0
1.5
1.0
0.5
0.0
ΔF/F0
OV2008 (DMSO)
OV2008 (CDDP)
C13* (DMSO)
C13* (CDDP)
***
OV2008
C13*
At 50 min.
0
10
20
30
40
50
Time (mins)
DMSO
CDDP
DMSO
CDDP

Figure 4.11

To test if the lack of CDDP-induced $[Ca^{2+}]_i$ increase in C13* cells may be attributed to a defect in the machinery of Ca^{2+} mobilization, increased $[Ca^{2+}]_i$ was induced artificially through cell membrane permeabilization using potassium chloride (KCl). KCl depolarizes the cell membrane by activating the voltage-operated Ca^{2+} channels (described in Chapter 1) that leads to an increase in the cytosolic free Ca^{2+}. The changes in the $[Ca^{2+}]_i$ caused by 35 mM of KCl were measured in both OV2008 and C13* cells using confocal microscopy as described earlier. KCl induced a dramatic increase in the $[Ca^{2+}]$i in both cell lines (Figure 4.12), indicating that Ca^{2+} mobilization from the extracellular space into these cells is functional and could unlikely be the reason behind the failure of CDDP to increase the $[Ca^{2+}]_i$ in C13* cells.

To further investigate the defect in the mechanism underlying Ca^{2+} mobility in C13* cells, the release of Ca^{2+} from the internal stores (*e.g.* endoplasmic reticulum; ER) was attempted by activating the ryanodine receptors (RyRs; described in Chapter 5) using 40 μM of caffeine in OV2008 and C13*. Unexpectedly, caffeine failed to increase the $[Ca^{2+}]_i$ in both cells (Figure 4.13) suggesting that either the RyRs are not expressed or functional at the ER membrane or Ca^{2+} mobilization from the internal stores is not effective in these cells.

The second possibility was addressed using a different strategy to release Ca^{2+} from the internal stores that does not involve the RyRs. Cyclopiazonic acid (CPA; 100μM) which is a selective Ca^{2+}-ATPase inhibitor, was employed to release Ca^{2+} by depleting the ER stores through blocking the opening of the Ca^{2+}-ATPase pump, and thus preventing Ca^{2+} entry to the ER stores (Moncoq, Trieber *et al.* 2007).

CPA caused a sharp increase in the $[Ca^{2+}]_i$ within a few seconds in both OV2008 and C13* cells as demonstrated in figure 4.14, excluding any defect in Ca^{2+} mobilization from the internal stores in C13*.

Figure 4.12: The effect of KCl on the $[Ca^{2+}]_i$ in OVCA cells.

KCl caused a sharp increase in the $[Ca^{2+}]_i$ in both chemosensitive (OV2008) and chemoresistant (C13*) cells. OV2008 and C13* cells were subjected to Ca^{2+} imaging where the effect of KCl on the $[Ca^{2+}]_i$ was assessed by confocal microscopy. Data represents four independent experiments at each time-point.

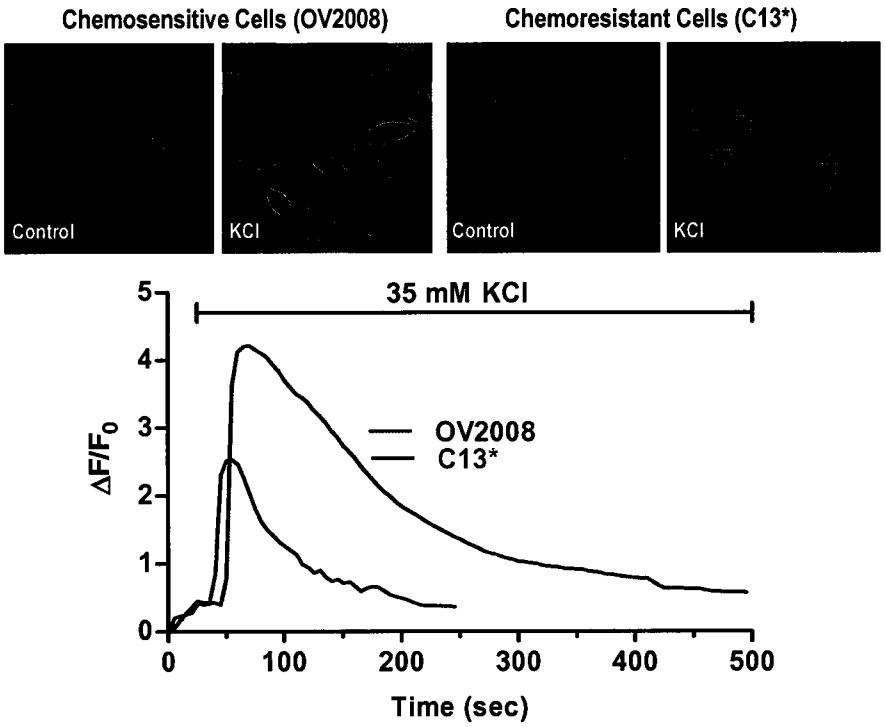

Chemosensitive Cells (OV2008)
Chemoresistant Cells (C13*)
Control
KCl
Control
KCl
35 mM KCl
OV2008
C13*
ΔF/F0
0
1
2
3
4
5
0
100
200
300
400
500
Time (sec)

Figure 4.12

Figure 4.13: The effect of caffeine on the $[Ca^{2+}]_i$ in OVCA cells.

Caffeine failed to increase the $[Ca^{2+}]_i$ in either of the tested cells. Chemosensitive (OV2008) and chemoresistant (C13*) cells were subjected to calcium imaging where the effect of caffeine on the $[Ca^{2+}]_i$ was assessed by confocal microscopy. Data represents four independent experiments at each time-point.

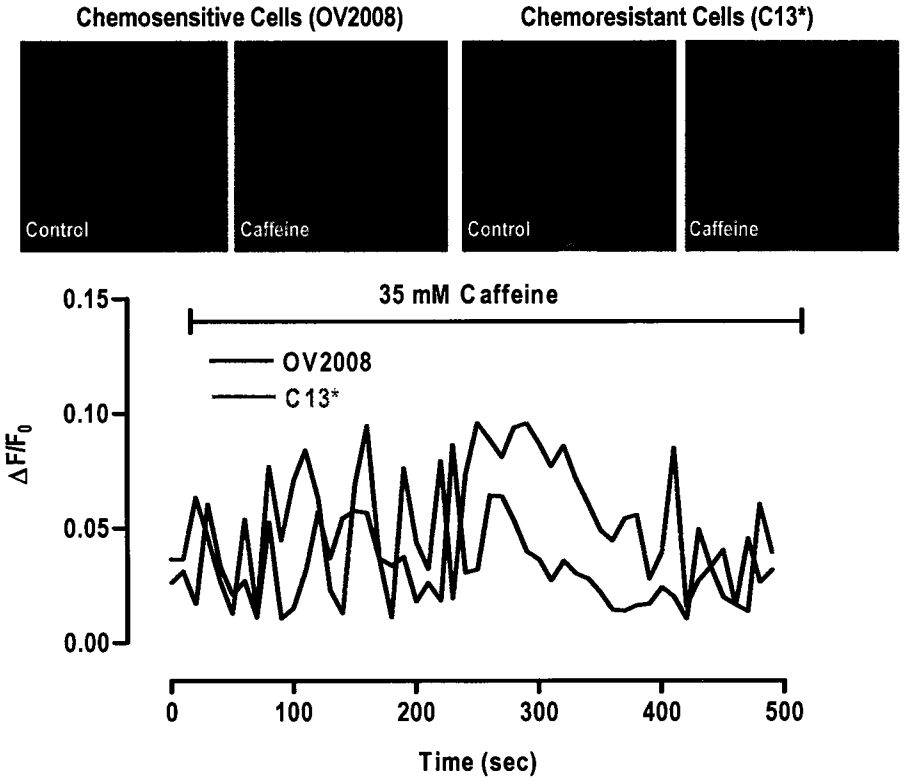

Chemosensitive Cells (OV2008)
Chemoresistant Cells (C13*)
Control
Caffeine
Control
Caffeine
35 mM Caffeine
OV2008
C13*
ΔF/F0
0.15
0.10
0.05
0.00
0
100
200
300
400
500
Time (sec)

Figure 4.13

Figure 4.14: The effect of CPA on the $[Ca^{2+}]_i$ in OVCA cells.

CPA induced a significant increase in the $[Ca^{2+}]_i$ in both chemosensitive (OV2008) and chemoresistant (C13*) cells. The effect of CPA on the $[Ca^{2+}]_i$ in OV2008 and C13* cells was assessed by confocal microscopy. Data are represented as the mean ± SEM of four independent experiments. Two way-ANOVA indicates a significant CPA effect ($P < 0.001$) and cell line effect ($P < 0.01$) on $[Ca^{2+}]_i$ and significant CPA-cell line interaction ($P < 0.05$). Bonferroni post hoc test shows significant differences between DMSO and CPA [$P < 0.001$] in both cell lines.

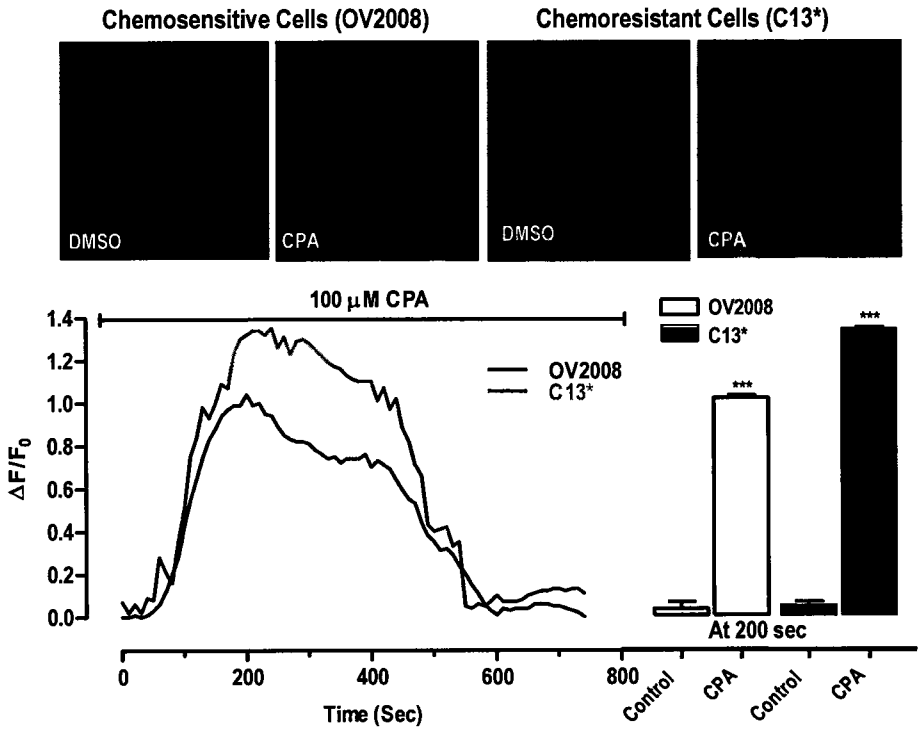

Chemosensitive Cells (OV2008)
Chemoresistant Cells (C13*)
DMSO
CPA
DMSO
CPA
100 μM CPA
OV2008
C13*
ΔF/F0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
Time (Sec)
OV2008
C13*
***
***
At 200 sec
Control
CPA
Control
CPA

Figure 4.14

4.8 Calcium is required for calpain activation, p73α down-regulation and apoptosis induction in ovarian cancer cells.

To determine if the inability of the C13* cells to undergo apoptosis in response to CDDP was related to the observed deregulation of CDDP-induced $[Ca^{2+}]_I$ increase, we examined the influence Cyclopiazonic acid (CPA; 100 μM) on calpain activation (illustrated by cleaved α-fodrin), TAp73α and ΔNp73α content and apoptosis in OVCA cells. OV2008 and C13* cells were treated with CPA (100 μM) or DMSO (control) and harvested at different time point (0, 6, 12 and 24 h). Protein contents and apoptosis were assessed by Western blot and Hoechst stain, respectively.

CPA significantly induced apoptosis in a time-dependent manner starting at 6 hours for OV2008 and 12 hours for C13* (Figure 4.15). It also activated calpain as evident by the presence of cleaved α-fodrin, resulting in TAp73α and ΔNp73α down-regulation/cleavage in both cell lines. Glucose regulated protein 78 [GRP78; an indicator of ER stress (Di Sano, Ferraro *et al.* 2006)] content increased consistently with the CPA-induced, calpain activation and apoptosis. These findings suggested that the defect is neither at the level of calpain nor p73, but rather in the $[Ca^{2+}]$ signalling in response to CDDP treatment. These observations also suggest that Ca^{2+} is required for calpain activation and subsequent TAp73α and ΔNp73α down-regulation/cleavage and apoptosis.

Figure 4.15: The effect of CPA on calpain activation, TAp73α and ΔNp73α content and apoptosis in OVCA cells.

CPA induced calpain activation, TAp73α and ΔNp73α down-regulation/cleavage and apoptosis in chemosensitive (OV2008) and chemoresistant (C13*) cells. Both OV2008 and C13* cells were treated with CPA (100 μM) or DMSO (control) and harvested at different time point (0, 6, 12 and 24 h). TAp73α, ΔNp73α, GRP78, cleaved α-fodrin and GAPDH contents and apoptosis were measured by Western blot and Hoechst stain, respectively. Data are represented as the mean ± SEM of three independent experiments. Two way-ANOVA indicates a significant CDDP effect ($P < 0.001$) and cell line effect ($P < 0.05$) and significant CDDP-cell line interaction ($P < 0.05$). Bonferroni post hoc test shows significant differences between DMSO and CDDP [$P < 0.05$ (OV2008 at 6 h) and $P < 0.001$ (OV2008 and C13* at 12 and 24 h)].

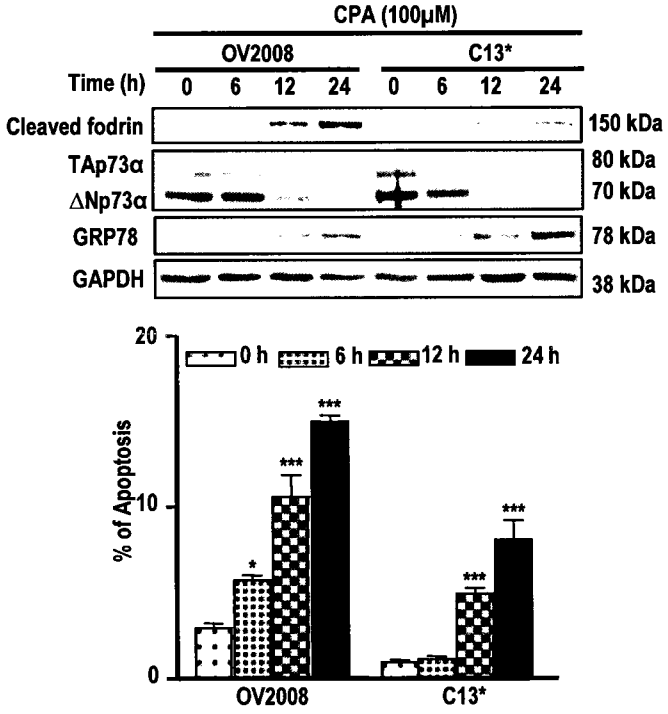

CPA (100μM)
OV2008
C13*
Time (h)
0 6 12 24 0 6 12 24
Cleaved fodrin
150 kDa
TAp73α
ΔNp73α
80 kDa
70 kDa
GRP78
78 kDa
GAPDH
38 kDa
0 h
6 h
12 h
24 h
% of Apoptosis
20
10
0
OV2008
C13*

Figure 4.15

4.9 CDDP-induced $[Ca^{2+}]_i$ increase involves mobilization of intracellular Ca^{2+} stores in chemosensitive cells

Although we showed that CDDP increased the $[Ca^{2+}]_i$ in OV2008 cells (Figure 4.11), the source (intracellular versus extracellular) of the Ca^{2+} is unknown. The possible contribution of the extracellular Ca^{2+} in the CDDP-induced $[Ca^{2+}]_i$ increase was assessed in OV2008 cells cultured in normal Ca^{2+} concentration (0.1 g/L) or Ca^{2+} -free media. Removal of extracellular Ca^{2+} (*e.g.* Ca^{2+}-free media) had no effect on the CDDP-induced $[Ca^{2+}]_i$ elevation when compared to the response observed with normal Ca^{2+} media (Figure 4.16). However, the increase in the $[Ca^{2+}]_i$ upon CDDP treatment in this media declined faster than in the regular media which was sustained up to the termination of the experiment. This decline could be explained by the lack of Ca^{2+} influx to replenish the internal stores (Putney, Broad *et al.* 2001).

To present additional confirmation that the CDDP-mediated $[Ca^{2+}]_i$ increase is mediated via mobilization of intracellular Ca^{2+} stores, internal Ca^{2+} stores in OV2008 were first depleted by CPA prior to CDDP treatment. Figure 4.17 demonstrated that CDDP failed to increase the $[Ca^{2+}]_i$ after intracellular Ca^{2+} store depletion in both regular (A) and Ca^{2+}-free (B) media over-time, indicating that the CDDP-induced $[Ca^{2+}]_i$ increase is dependent on these stores (C).

The effects of both CDDP and CPA were then extended to examine calpain activation and thus TAp73α and ΔNp73α down-regulation/cleavage and apoptosis induction in both OV2008 and C13* cells. These cells were treated with CDDP (10 μM) and/or CPA (100 μM) and harvested 24 hours post treatment. TAp73α, ΔNp73α, GRP78, cleaved α-fodrin and GAPDH contents and apoptosis were measured by Western blot and Hoechst stain, respectively.

Figure 4.18 showed that CDDP induced calpain activation (illustrated by the presence of cleaved α-fodrin), TAp73α and ΔNp73α down-regulation/cleavage and apoptosis in OV2008 cells but not in C13*, whereas CPA caused such responses in both cell lines. While CDDP did not enhance CPA-dependent calpain activation and apoptosis in C13* cells, it had a significant effect on these events in OV2008. These findings suggested that CPA alone may only increase the $[Ca^{2+}]_i$, activate calpain and induce apoptosis in C13* whereas the CDDP could also activate Ca^{2+}-independent pathways to facilitate these outcomes in OV2008 cells.

Figure 4.16: The effect of CDDP on the $[Ca^{2+}]_i$ of chemosensitive cells cultured in regular and Ca^{2+}-free media.

CDDP induced $[Ca^{2+}]_i$ increase was not affected by removal of extracellular Ca^{2+} (*i.e.* Ca^{2+}-free media). OV2008 cells were cultured in both regular and Ca^{2+}-free media and the effect of CDDP on $[Ca^{2+}]_i$ were measured as described in Chapter 3. Data are represented as the mean ± SEM of four independent experiments. Two way-ANOVA indicates a significant CDDP effect ($P < 0.001$) and media effect ($P < 0.05$) on $[Ca^{2+}]_i$. Bonferroni post hoc test shows significant differences between DMSO and CDDP [$P < 0.001$] in both regular and Ca^{2+}-free media.

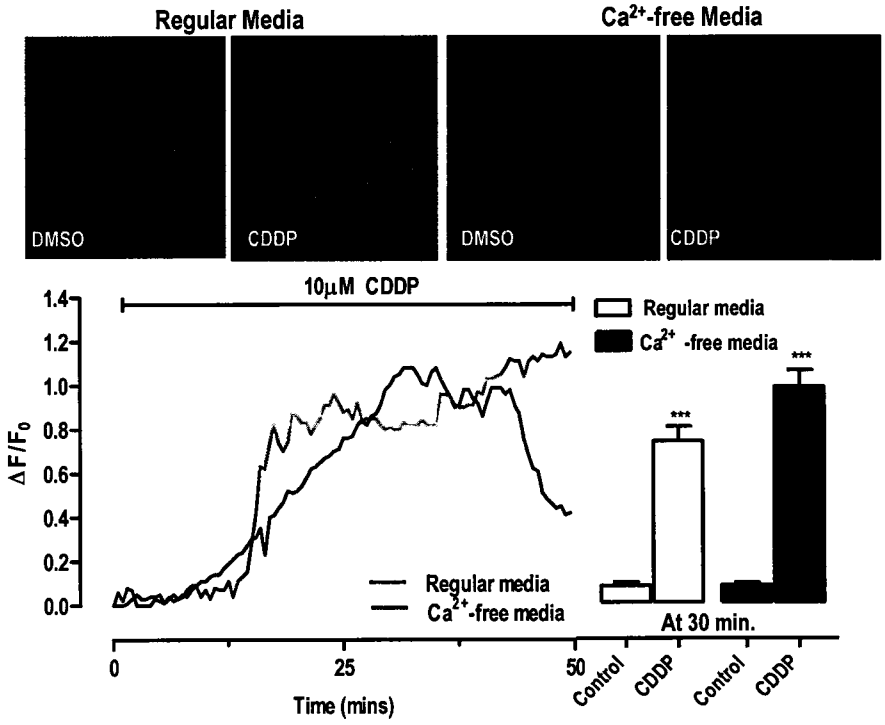

Regular Media
Ca2+-free Media
DMSO
CDDP
DMSO
CDDP
10μM CDDP
Regular media
Ca2+ -free media
***
***
ΔF/F0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Regular media
Ca2+-free media
At 30 min.
0
25
50
Time (mins)
Control
CDDP
Control
CDDP

Figure 4.16

Figure 4.17: The effect of CDDP and CPA on the $[Ca^{2+}]_i$ of chemosensitive cells cultured in regular and Ca^{2+}-free media.

CDDP-induced $[Ca^{2+}]_i$ increase was abolished after intracellular Ca^{2+} store depletion by CPA in both **A)** regular and **B)** Ca^{2+}-free media. OV2008 intracellular Ca^{2+} stores were depleted by CPA prior to CDDP treatment over time and changes in $[Ca^{2+}]_i$ were assessed as mentioned in Chapter 3. **C)** Fluorescent images were obtained before and after the addition of either CPA alone or with CDDP. Data are represented as the mean ± SEM of four independent experiments at each time-point. Three way-ANOVA indicates a significant CPA effect ($P < 0.001$) and CDDP effect ($P < 0.001$) on $[Ca^{2+}]_i$ and significant CPA-CDDP interaction ($P < 0.01$) in both regular and Ca^{2+}-free media. Bonferroni post hoc test shows significant differences between DMSO and CPA [$P < 0.001$] and CDDP [$P < 0.001$] in both media.

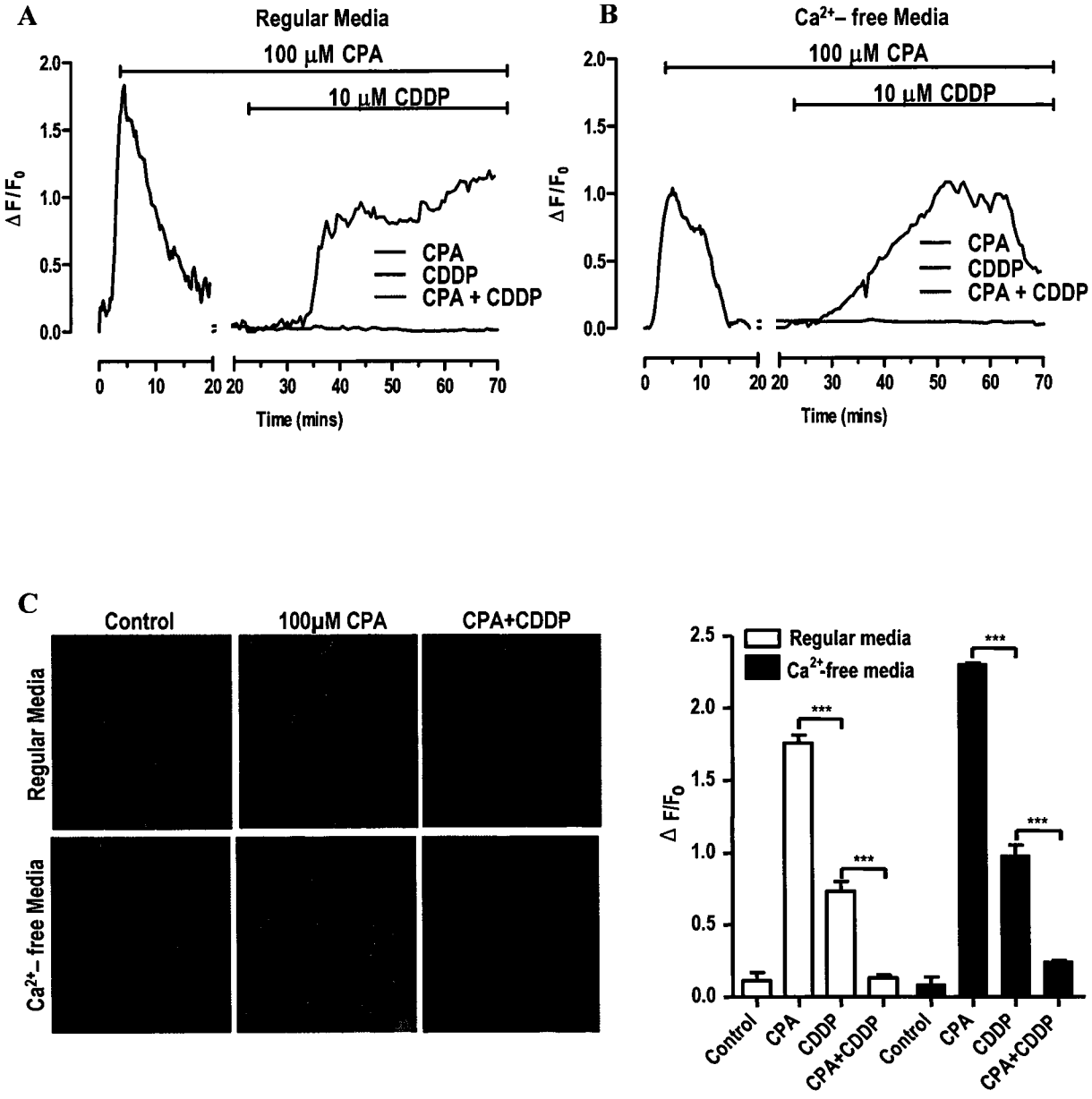

A
Regular Media
100 μM CPA
10 μM CDDP
CPA
CDDP
CPA + CDDP
ΔF/F0
Time (mins)
B
Ca2+– free Media
100 μM CPA
10 μM CDDP
CPA
CDDP
CPA + CDDP
ΔF/F0
Time (mins)
C
Control
100μM CPA
CPA+CDDP
Regular Media
Ca2+– free Media
Regular media
Ca2+-free media
Δ F/F0
Control
CPA
CDDP
CPA+CDDP
Control
CPA
CDDP
CPA+CDDP

Figure 4.17

Figure 4.18: The effect of CDDP and CPA on calpain activation, TAp73α and ΔNp73α content and apoptosis in OVCA cells.

CDDP induced calpain activation, TAp73α and ΔNp73α down-regulation/cleavage and apoptosis in chemosensitive (OV2008) but not chemoresistant (C13*) cells while CPA caused such responses in both cell lines. Both OV2008 and C13* cells were treated with CDDP (10 μM) and/or CPA (100 μM) and harvested 24 hours post treatment. TAp73α, ΔNp73α, GRP78, cleaved α-fodrin and GAPDH contents and apoptosis were measured by Western blot and Hoechst stain, respectively. Data are represented as the mean ± SEM of three independent experiments. Three way-ANOVA indicates a significant CPA effect ($P < 0.001$) and CDDP effect ($P < 0.001$) on apoptosis$_i$ and significant CPA-CDDP interaction ($P < 0.01$) in both regular and Ca^{2+}-free media. Bonferroni post hoc test shows significant differences between DMSO and CPA [$P < 0.001$] and CDDP [$P < 0.001$ (OV2008 and C13*)] and CPA-CDDP [$P < 0.001$(OV2008)].

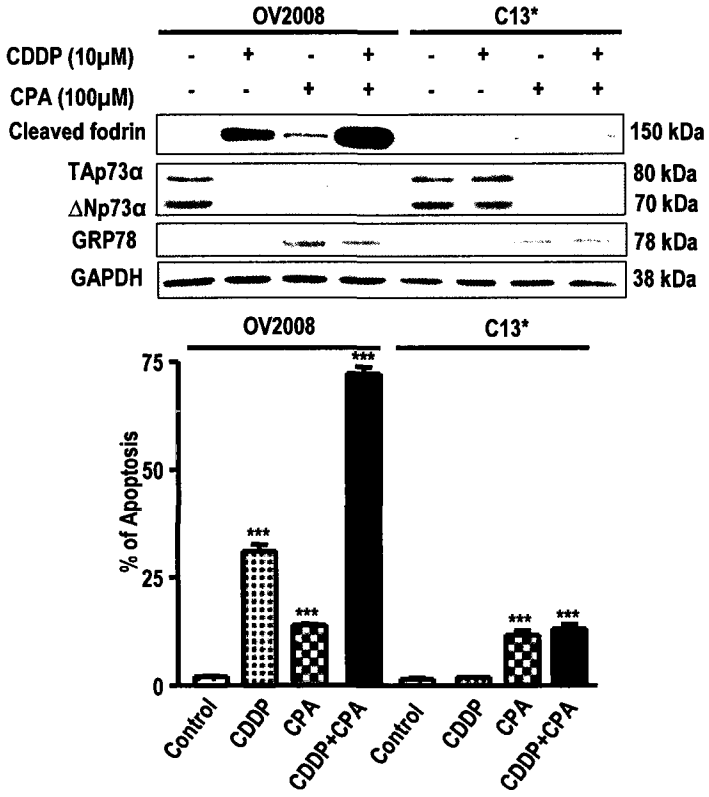

OV2008
C13*
CDDP (10μM)
CPA (100μM)
Cleaved fodrin
150 kDa
TAp73α
ΔNp73α
80 kDa
70 kDa
GRP78
78 kDa
GAPDH
38 kDa
OV2008
C13*
% of Apoptosis
75
50
25
0
***
Control
CDDP
CPA
CDDP+CPA

Figure 4.18

CHAPTER 5: DISCUSSION

5.1 Overview and Significance

Chemoresistance is a major concern for cancer chemotherapy, remaining an obstacle for the successful treatment of ovarian cancer. Although CDDP is an effective anti-cancer drug used for OVCA, its efficacy is limited by the development of resistance. While chemoresistance may be attributed to altered DNA repair, drug transport and metabolism, CDDP resistance in OVCA is also associated with defects in drug-induced apoptosis. This is a result of the up-regulation and the reduction of either the pro- or anti-apoptotic factors.

The current thesis addresses some of the vital regulatory mechanisms involved in CDDP-induced apoptosis and their contribution to OVCA cell sensitivity. The goal of this thesis was to examine the role of three critical regulators in CDDP-induced apoptosis in OVCA cells: calcium (Ca^{2+}), calpain and p73α, as well as how their deregulation related to CDDP resistance. The strategies adopted by this thesis allowed for the modulation of the content or the function of these regulators to study CDDP-induced apoptosis in OVCA cells.

Initially, we examined the role of p73α in the regulation of OVCA cell sensitivity to CDDP. This was achieved by manipulating the p73α gene product, using over-expression and down-regulation strategies. Secondly, we assessed one of the p73α recently identified up-stream mediators, calpain, to determine how such a mediator regulated the steady state level of p73α in CDDP-induced apoptosis. The effect of CDDP on calpain activation and how such activation might influence CDDP-induced apoptosis was also evaluated. We investigated the effect of CDDP on the intracellular Ca^{2+}

concentrations $[Ca^{2+}]_i$ and its mobilization. In addition, calpain activation and p73α-mediated apoptosis in OVCA cells was characterized. Collectively, these studies highlighted an important link between these regulators that are influenced by CDDP and result in apoptosis induction: any defect in this link was enough to confer CDDP-resistance. Understanding the molecular basis of the CDDP-induced, calpain-mediated p73α processing pathway may direct future research for the treatment of certain chemoresistant OVCA.

5.2 Experimental Advantages of Matched Pair Ovarian Cancer Cell Lines

The present thesis had the advantage of using two matched pairs of chemosensitive parental cell lines (OV2008 and A2780s) with their isogenic resistant counterparts (C13* and A2780cp). The chemosensitive cells were obtained from serous cystodenocarcinoma cells (DiSaia, Sinkovics *et al.* 1972). Their resistant variants were developed through extended cultures in the presence of increasing concentrations of CDDP (Brown, Clugston *et al.* 1993; Mamenta, Poma *et al.* 1994). The chemosensitive and chemoresistant cells share the same genetic background, excepting those factors that determine chemosensistivity and have been changed during the development of the resistant variant.

The utilization of these cell pairs allowed for appropriate conclusions regarding the observed differences between the chemosensitive and chemoresistant cells in regards to the effect of TAp73α and ΔNp73α over-expression on CDDP-induced apoptosis. The between-group differences yielded predictable outcomes that were not due to the random differences between cell types. In addition, the findings gathered are more relevant and applicable for the study of chemoresistance. Although two pairs were used for the over-

expression experiment, the lack of p73 expression in A2780s cells due to CpG island hypermethylation (Chen, Ip *et al.* 2000) (Chapter 4; Figure 4.1C) was the justification for not using this pair in subsequent studies.

5.3 Deregulation of Apoptosis and Cisplatin Resistance

It is well known that carcinogenesis occurs via the activation of abnormal cell growth and suppression of apoptosis and therefore, many of the anti-cancer agents currently used (*e.g.* CDDP) are designed to modulate these events in cancer cells (Kaufmann and Gores 2000). CDDP-induced apoptosis is crucial for the successful treatment of OVCA although CDDP recently has been repaced by carpoplatin in some countries such as Canada (Canadian Cancer Society, 2009); any abnormal regulation of this signaling pathway has been correlated with a poor outcome (Gibb, Taylor *et al.* 1997; Shimada, Kigawa *et al.* 2000; Bardella, Dettori *et al.* 2007). For this reason, modulating the critical regulators in this apoptotic mechanism should be an essential factor in overcoming CDDP-resistance in OVCA.

For this thesis, CDDP-induced apoptosis in the matched pairs of OVCA cells were compared. In both a time- and a concentration-dependent manner (Chapter 4; Figure 4.1A and B), we found that CDDP induced apoptosis in the chemosensitive OVCA cells (OV2008 and A2780s), but not in their resistant counterparts (C13* and A2780cp). These findings are consistent with the previously published results from our laboratory, suggesting that apoptosis induction is a vital determinant of OVCA cell sensitivity to CDDP (Asselin, Mills *et al.* 2001; Abedini, Qiu *et al.* 2004; Fraser, Bai *et al.* 2008).

Practically, apoptosis can be assessed by different methods, which may vary in their sensitivity and specificity. Our laboratory routinely measured CDDP-induced apoptosis based upon cellular and nuclear morphology. The Hoechst stain was used where a minimum of four hundred cells per treatment group were counted. Selected fields and blinded slides were tested randomly to avoid experimental bias (Sasaki, Sheng *et al.* 2000; Asselin, Mills *et al.* 2001; Fraser, Leung *et al.* 2003; Abedini, Qiu *et al.* 2004; Fraser, Chan *et al.* 2006; Yan, Fraser *et al.* 2006; Yang, Fraser *et al.* 2006; Abedini, Muller *et al.* 2008; Fraser, Bai *et al.* 2008; Yang, Fraser *et al.* 2008; Abedini, Muller *et al.* 2009). Hoechst stain is a well-established and accepted method used to detect morphological features of the apoptotic cell nuclei, such as nuclear shrinkage, fragmentation and chromatin condensation, thus satisfying our experimental parameters as a suitable technique (Liang, Zhang *et al.* 2004; Yang, Kuo *et al.* 2005; Wang, Mao *et al.* 2007; Wang, Liu *et al.* 2008; Shan, Sheng *et al.* 2009).

Further experiments lead us to examine caspase-3 activation, illustrated by its well-known substrate (*i.e.* PARP) cleavage on Western blot. This was an additional conformation of the CDDP-induced, caspase-mediated apoptosis. In figure 4.3, CDDP-induced apoptosis mediated by TAp73α and ΔNp73α over-expression was assessed by both Hoechst staining and caspase-3 activation (illustrated by PARP cleavage), to confirm its accuracy as a measurement.

5.4 Regulation of P73 in Cisplatin-Induced Apoptosis

Although our laboratory and others have extensively studied the role of p53 in CDDP-induced apoptosis in OVCA and how its deregulation might contribute to CDDP-resistance, p73 had yet to receive such attention (Kanamori, Kigawa *et al.* 1998; Mujoo, Zhang *et al.* 2000; Shimada, Kigawa *et al.* 2000; Kigawa, Sato *et al.* 2002; Fraser, Leung *et al.* 2003; Abedini, Qiu *et al.* 2004; Fraser, Chan *et al.* 2006; Yan, Fraser *et al.* 2006; Yang, He *et al.* 2006; Yang, Fraser *et al.* 2006; Horiuchi, Wang *et al.* 2007; Fraser, Bai *et al.* 2008; Abedini, Muller *et al.* 2009).

To our knowledge, we are the first to investigate the role of endogenous p73α in CDDP-induced apoptosis in OVCA cells in the context of CDDP-resistance. Here, we showed that TAp73α over-expression significantly enhanced CDDP-induced apoptosis in different OVCA cell lines (Chapter 4; Figure 4.3). The effect of ΔNp73α over-expression was variable, as well as ΔNp73β and ΔNp73γ over-expression, which showed equivalent trends on CDDP-induced apoptosis (Chapter 4; Figure 4.4). We also demonstrated that p73α down-regulation attenuated CDDP-induced, PUMA and NOXA up-regulation and apoptosis in chemosensitive OVCA cells (Chapter 4; Figure 4.5). Based on our findings, endogenous TAp73α is a crucial mediator in CDDP-induced apoptosis in certain OVCA cells. ΔNp73α-mediated apoptosis was found to be cell-specific and/or dependent upon the relative importance of specific regulators in different cell lines.

Of the few reports published on the role of p73 in ovarian cancer and CDDP treatment, only the effect of exogenous p73 on either the activation of down-stream genes and cell growth or the effect of CDDP on the endogenous level of p73 have been reported (Vikhanskaya, D'Incalci *et al.* 2000; Vikhanskaya, Marchini *et al.* 2001; Muscolini, Cianfrocca *et al.* 2008; Righetti, Perego *et al.* 2008). Vikhanskaya *et al.* addressed the consequences of over-expressing p73α in wild-type p53-expressing human OVCA (A2780) cells (Vikhanskaya, D'Incalci *et al.* 2000). These investigators found that p73α competed with p53, and could modulate its function by inhibiting p53 DNA-binding. They also showed that CDDP had no effect upon the p73α content. Since these cells were stably transfected with p73α, the effect of CDDP could be underestimated due to the saturation of the cell system with p73α.

A year later, the same group examined the consequences of p73α over-expression on the gene expression and the cellular response to CDDP in the same cell lines (Vikhanskaya, Marchini *et al.* 2001). They found that p73α over-expression was associated with the up-regulation of genes encoded for DNA repair proteins and CDDP resistance. These results differ from our findings, which showed TAp73α over-expression enhanced basal apoptosis and CDDP-induced apoptosis in OVCA cells. We believe our approach of measuring CDDP-induced apoptosis by Hoechst stain and capsase-3 activation to be more specific for apoptosis count than the clonogenic assay used in the 2001 study, where only the number of colonies that survived CDDP treatment was counted. The inhibition of growth as determined by this assay does not always represent apoptosis, since necrosis and inhibition of cell proliferation can not be excluded as a source of experimental error. Commonly, p73 over-expression can lead to the up-

regulation of DNA repair proteins and p73 has been shown to be involved in DNA repair pathways (Musat, Vax *et al.* 2004; Lin, Sengupta *et al.* 2009). These studies did not determine if the endogenous p73 contributed to CDDP-resistance in ovarian cancer cells.

Recently, a study reported that CDDP slightly increased the p73 content in chemosensitive OVCA (A2780) cells, but not in its resistant counterpart (A2780/BBR3464), the former of which was associated with substantial apoptosis (Righetti, Perego *et al.* 2008). In this context, another group found that CDDP significantly increased the p73α content in chemosensitive OVCA (A2780) cells, but not in its resistant derivative (A2780 CIS) (Muscolini, Cianfrocca *et al.* 2008). They also demonstrated that pretreatment of resistant A2780 CIS cells with Trichostatin A (histone deacetylase inhibitor) overcame apoptosis resistance to CDDP by up-regulating the p73α content in these cells. Both groups, however, used much higher concentrations of CDDP (25-50 μM) compared with the standard concentration (10 μM) for OVCA treatment that has been used in the work of this thesis.

In our system, the expression of TAp73α and ΔNp73α and the effect of CDDP on their contents in the tested OVCA cells were variable. We showed that CDDP decreased both isoform contents in chemosensitive (OV2008) cells, but not in its resistant counterpart (C13*). TAp73α was not expressed in A2780s cells. This was attributed to the CpG island hypermethylation (Chen, Ip *et al.* 2000), where the 5-Aza Deoxycitidine, a demethylating agent, was used to restore its expression (Chapter 4; Figure 4.1C). In contrast, CDDP up-regulated the TAp73α content in chemosensitive (OVCA 432) and chemoresistant (A2780cp) OVCA cells and Hey cells (Chapter 4; Figure 4.1B & 4.5C, respectively). Although these two cell lines respond differently to CDDP, both harbor

mutant p53. We speculate that TAp73α may act as a supplemental protein for p53 function: when the p53-dependent pathway is deregulated, CDDP may utilize the TAp73α-dependent pathway to trigger apoptosis. Further investigations are required to examine this hypothesis, including the restoration of functional p53 in such cells to detect the effect of CDDP on TAp73α content.

In contrast to TAp73α expression in ovarian cancer cells, ΔNp73α protein content was only detected in OV2008, yet its mRNA abundance was identified in all tested cell lines. We proposed that the lack of ΔNp73α protein content in C13*, A2780s and A2780cp cells could be due to a defect in TAp73/p53-dependent transcriptional activity. In other words, TAp73 isoforms and p53 have been shown to directly activate the transcription of ΔNp73 isoforms through a specific DNA element in its promoter (Kartasheva, Contente et al. 2002; Nakagawa, Takahashi et al. 2002). For instance, ΔNp73α content was high in OV2008 cells, which we believe harbour functional TAp73, unlike A2780s that did not express TAp73α (Chapter 4; Figure 4.1C). Regarding the loss of ΔNp73α protein in chemoresistant (C13* and A2780cp) cells, we speculate that the TAp73 isoform may be non-functional in these cells. Although, CDDP up-regulated TAp73α content in A2780cp, the presence of mutant p53 could inhibit TAp73 activity (Strano, Munarriz et al. 2000; Bergamaschi, Gasco et al. 2003).

The chemoresistant (C13* and A2780cp) cells were also found to be deficient in functional p53, which has been demonstrated recently by our laboratory (Fraser, Bai *et al.* 2008). Further investigations are required to test this hypothesis, including the restoration of functional TAp73 in A2780s cells, or activating p53 and/or TAp73α in the chemoresistant cells.

In addition to the previously published studies, our findings provided a better insight on the role of the endogenous p73α in CDDP-induced apoptosis in OVCA cells.. Using two pair of OVCA cell lines for the over-expression experiment and specific cDNA for each isoform allowed us to differentially assess their effect on CDDP-induced apoptosis. Furthermore, the down-regulation approach provided us with further evidence that the endogenous p73α can contribute to OVCA cell sensitivity to CDDP. One potential disadvantage for the use of siRNA to down-regulate both TAp73α and ΔNp73α is the inability to define the specific p73α involved. A specific reagent is difficult to prepare due to the high level of homology between the p73 isoforms. To overcome this deficiency, a dominant negative inhibitor can be introduced to the system to specifically inhibit the ΔNp73α, but not the TAp73α.

The differential role of p73 and its regulation by CDDP in different OVCA cells and in other cancer cells directs to the notion that p73 regulation by CDDP is restricted by certain parameters (Ono, Nonomura *et al.* 2001; Furuya, Ozaki *et al.* 2007; Sabatino, Previdi *et al.* 2007; Yoshida, Ozaki *et al.* 2008; Marrazzo, Marchini *et al.* 2009; Sang, Ando *et al.* 2009). Several theories have been proposed, including theories of the dependence upon the relative distribution of p73 isoforms and theories of the distribution of p73 family members (p53 and p63) within the cell and the surrounding tissues. Specifically, the selective activation by the up-stream activators of those family members will depend upon abundance and/or location. Other theories depend upon the origin of the cell type or the specificity that is controlled by internal mechanisms.

A recently published paper supported these theories; the researchers found that the effect of TAp73 isoforms on the cell cycle and apoptosis is cell-specific (Holcakova, Ceskova et al. 2008).

Due to the presence of a complex network of p73 isoforms, other family member's expression and function and their mutual influence on p73, presents experimental challenges that are predictable when studying p73. Selective and specific approaches such as specific antibodies and/or inhibitors for each isoforms should be developed to precisely assess the role of p73 in CDDP-induced apoptosis in ovarian cancer cells.

5.5 The Role of Calcium-Mediated Calpain Pathway

Although CDDP targets DNA, extra-nuclear targets, such as the endoplasmic reticulum, have also been identified (Mandic, Hansson *et al.* 2003). CDDP induced caspase-3 activation and apoptosis in enucleated cells (cytoplasts) were found to cause ER stress and subsequently, Ca^{2+} was released and calpain activated. In this context, the current thesis demonstrated that CDDP induced an increase in $[Ca^{2+}]_i$ and in calpain activation (illustrated by α-fodrin cleavage) in the chemosensitive (OV2008), but not in its resistant counterpart (C13*) cells (Chapter 4; Figures 4.9 and 4.11). Interestingly, we also found that Cyclopiazonic acid (CPA), a selective Ca^{2+}-ATPase inhibitor, promoted such a response, as well as p73α down-regulation and apoptosis in both cell types (Chapter 4; Figures 4.14 and 4.15). The elevation of CDDP-induced $[Ca^{2+}]_i$ in OV2008 cells was not effected by the elimination of extracellular Ca^{2+}, but it was abolished by the depletion of the internal Ca^{2+} stores (Chapter 4; Figures 4.16 and 4.17). This indicated that the internal Ca^{2+} stores were likely involved. These observations suggest that the deregulation of Ca^{2+} signaling, but not of calpain or p73 in response to CDDP, may be a key factor that may contribute to CDDP resistance in the C13* cells. Ca^{2+} is required for calpain activation, and subsequently, TAp73α and ΔNp73α processing and apoptosis in this cell line pair.

While another study showed that calpain cleaves p73 isoforms at both the N- and the C-terminus (Munarriz, Bano *et al.* 2005), we are the first to demonstrate that calpain regulates TAp73α and ΔNp73α content in the CDDP-induced apoptosis of cancer cells. Calpain inhibition by a specific inhibitor (Calpeptin) and/or by RNA silencing is strong evidence of the involvement of calpain in the CDDP-induced, TAp73α and ΔNp73α

processing in OV2008 cells. Moreover, the effect of CDDP on calpain/p73α interactions and the co-localization in these cells also provided further support for such involvement. This may explain calpain's regulation of TAp73α and ΔNp73α content.

The consensus site of calpain cleavage in the p73 sequence is unknown. Due to the variations between the p73 isoforms at both termini, where calpain cleavage sites are present, it is currently difficult to construct a mutant p73 that lacks these sites. This would be necessary in order to study the functional outcome of calpain-mediated, p73 cleavage without confounding variables. Further work is required to identify these terminal sites, including sequence recognition of the cleaved fragments that would be mutated by site-directed mutagenesis.

It is not known whether or not calpain-mediated cleavage would result in p73α degradation, as in the case of Xiap (Kobayashi, Yamashita *et al.* 2002) or Bax activation (Toyota, Yanase *et al.* 2003). We believe that such cleavage could lead to p73α activation because it is only present in the chemosensitive cells. We showed that both TAp73α and ΔNp73α over-expression enhanced CDDP-induced apoptosis; their down-regulation attenuated such a response in these cells.

The conflicting findings of calpain function, either as an apoptotic suppressor or promoter (described in Chapter 1), was expected and observed in CDDP-induced apoptosis. Mlynarczuk-Bialy *et al.* demonstrated that calpain inhibition induced apoptosis in CDDP resistant melanoma cells (Mlynarczuk-Bialy, Roeckmann et al. 2006). Liu *et al.* showed that calpain mediates CDDP-induced apoptosis by the activation of Bid, which regulated the mitochondrial-dependent apoptosis in human lung adenocarcinoma cells (Liu, Xing et al. 2008).

On the other hand, a study reported that the protection of auditory hair cells and neurons from CDDP-induced apoptosis was limited with calpain inhibition, suggesting that the calpain pathway was not involved. Taken together, these findings suggest that calpain-mediated, CDDP-induced apoptosis could be cell-specific. At this time, it is unknown if the difference in calpain-induced apoptosis is isoform-specific, given the wide range of calpain isoforms and the lack of specific inhibitors for each isoform. Only calpain 1, 2 and 4 are ubiquitously expressed isoforms, while the remaining twelve isoforms are tissue-specific. Further investigation is required to fully understand the precise role of each isoform in CDDP-induced apoptosis.

To the best of our knowledge, we are the first to show that calpain is required for CDDP-induced apoptosis in OVCA cells. Our findings revealed that the inhibition of calpain by calpeptin and siRNA attenuated CDDP-induced apoptosis in chemosensitive (OV2008) cells. This evidence is in favour of calpain as a promoter of CDDP-induced apoptosis. The activation of calpain by CDDP in these chemosensitive cells, but not in their resistant variants, provided further evidence for calpain's role in CDDP-induced apoptosis. Our studies support the notion that calpain-mediated apoptosis is cell-, isoform- and drug-specific; therefore, caution should be exercised when studying its role in CDDP-induced apoptosis in cancer cells. We can not ignore the presence of the fifteen known isoforms of calpain that may potenially be expressed as functionally different and independent proteins. We speculate that up-stream mediators, such as calcium, in terms of magnitude and spatial location, also influence the regulation of the calpain isoform activity.

Our findings of CDDP-induced $[Ca^{2+}]_i$ corroborate the above hypothesis. Specifically, changes in Ca^{2+} homeostasis in these cells partially confer CDDP resistance, as evidenced by the lack of $[Ca^{2+}]_i$ response in the chemoresistant (C13*) cells. Identifying the sources by which CDDP induces such a response could elucidate the underlying mechanism of CDDP's action and its deregulation in chemoresistance. Our results suggested that the ER could be a likely source for the CDDP-induced $[Ca^{2+}]_i$ increase. Further investigations are required to confirm the mediators of this process.

A recent study showed that the CDDP-induced $[Ca^{2+}]_i$ increase in HeLa cells was dependent upon extracellular Ca^{2+} and was mediated by the IP_3 receptors on cell membranes (Splettstoesser, Florea *et al.* 2007). Although we showed that the sources for the CDDP-induced $[Ca^{2+}]_i$ increase in one OVCA cell line was intracellular, it would be significant to determine if and how CDDP could induce the activation of the IP_3 receptors or of other receptors present on the ER membrane, such as SERCAs (described in Chapter 1). Electrophysiological experiments to measure the effect of CDDP on the ER membrane conductance and IP_3 activation could achieve such results (Splettstoesser, Florea *et al.* 2007). Although caffeine failed to increase the $[Ca^{2+}]_i$ in OV2008 and C13* cells (Chapter 4; Figure 4.13), ryanodine receptors (RyRs), should not be excluded as potential mediators of the CDDP-induced $[Ca^{2+}]_i$ increase. Other activators of these receptors could be used, for instance, Suramin (agonist of RyRs), in order to confirm the caffeine-mediated effect; their expression could also be revealed by Western blot in OVCA cells.

Generalizing one therapeutic strategy for the calpain-mediated pathway is difficult due to the above mentioned complications. Calpain activation has been identified as a common event in a variety of neurological diseases and interest in developing its inhibitors as a therapeutic approach has gained momentum (Carragher 2006). In contrast, others like Liu, Xing *et al.* would like to introduce calpain activation as a therapeutic strategy to overcome CDDP resistance in certain cancer cells (2008). We believe that the Ca^{2+}-mediated, calpain pathway is unique, and that it should be studied under cell- and -isoform specific conditions to be evaluated precisely for each disease.

5.6 Future Directions

The current thesis has provided definitive evidence that p73α regulates CDDP-induced apoptosis via the Ca^{2+}/calpain pathway in OVCA cells; this pathway is involved in determining OV2008 cell line sensitivity to CDDP *in vitro*. *In vitro* studies should be extended to include *in vivo* experiments with animal models, to be later validated by human clinical samples.

5.6.1 Animal Model

Despite the significant demonstration that the calpain/p73α pathway is involved in CDDP-induced apoptosis *in vitro* and that it is a contributor in the regulation of OV2008 cell line sensitivity, it will be essential to examine if this pathway is operational *in vivo.* This would determine if changes in this pathway would result in the inhibition of tumour growth. Xenografts of chemosensitive and resistant OVCA cells could be established in female Swiss nude mice as described previously (Shaw, Senterman *et al.* 2004). With the exception of C13*, the cell lines used in the *in vitro* experiments form tumours following intraperitoneal (IP) injection and respond to chemotherapeutic agents *in vivo* (Shaw, Senterman *et al.* 2004). CDDP (1.5 mg/kg, IP) administration and changes in tumour volume, apoptosis (TUNEL), Calpain content, cleaved α-fodrin (indicative of calpain activation) and p73α content and location could be determined by Western blot and immunohistochemistry. We expect that CDDP would induce apoptosis and would suppress tumour growth, as well as calpain activation (cleaved α-fodrin) and thus, suggest the occurrence of p73α cleavage.

Although gene delivery by adenovirus for the transiently stable transfected cell lines is a well-established protocol in our laboratory, the Tet-on regulated gene expression system (BD Biosciences) would be a better method. This is due to its efficiency and longer-lasting gene expression, in comparison with the transient transfection protocol.

In this system, puromycin and hygromycin sensitivity in chemoresistant cells would need to be determined. Then, these cells could be stably transfected with a vector containing the reverse tetracycline-controlled transactivator (rtTA) under the control of the cytomegalovirus (CMV) promoter. Selection would be made based on puromycin resistance. The clones obtained would be analysed for rtTA mRNA content by RT-PCR. Calpain and p73α isoforms cDNA and/or shRNA plasmids would be sequenced using construct-specific sequencing primers. Inserts could be cloned into a plasmid, where expression is controlled by the Tet-responsive element (TRE). Cells would be stably transfected with these plasmids, selected by hygromycin/puromycin resistance. Xenografts containing these stably transfected cells would be established as above.

Calpain and p73α over-expression or down-regulation would be induced *in vivo* by adding doxycycline to the drinking water. When tumours developed, the mice would be treated with CDDP. Tumour volumes and apoptosis would be recorded. We predict that Calpain and/or p73α over-expression would sensitize chemoresistant cells to CDDP. This would result in decreased tumour volume and enhanced CDDP-induced apoptosis, when compared with control cell-derived tumours. Calpain and/or p73α down-regulation would be expected to attenuate such responses.

5.6.2 Clinical Samples

Once the functional role of calpain and p73α in the regulation of CDDP sensitivity in OVCA cell has been established both *in vitro* and in a xenograft model, its clinical relevance and applicability to the *in vivo* human situation should be characterized. Clinical samples normally retain certain features of the original tumour that might be lost in cultured cells during their continuous passage. In order to achieve this goal, primary cultures of OVCA cells from ascities fluid and solid tumours from patients would require assessment.

For a proposed clinical trial, the effect of CDDP on calpain and p73α isoform contents (Western) as well as apoptosis induction (Hoechst 33258 and Caspase-3 and PARP cleavage) in primary cells should be determined using the fluid ascities before and during CDDP treatment and tumour recurrence. Whether or not calpain and p73α isoform levels are correlated with positive clinical outcomes would be examined. We predict that the calpain and the p73α isoform protein content and apoptosis induction would be up-regulated during the CDDP-responsive phase; however, their level and apoptosis induction would be expected to decrease during the CDDP-resistant phase. Forced expression of calpain and of p73α in cells obtained from recurrent tumours should result in CDDP-induced apoptosis. If both calpain and p73α are significant determinants, we expect that their over-expression would sensitise the cells isolated from the fluid ascities to CDDP-induced apoptosis.

For solid tumour tissues, mRNA and/or protein expression and localization of both calpain and p73α would be measured by *in situ* hybridization (ISH) and immunohistochemistry (IHC), respectively. Due to the cellular heterogeneity of these tumours, calpain and p73α expression could vary, thus explaining the differential effect of CDDP on TAp73α and ΔNp73α contents *in vitro.* It will be also clinically significant to correlate such expressions with the patients' survival outcome, thereby indicating the role of these proteins in tumour progression. We expect that the calpain and TAp73α protein contents would be up-regulated during the CDDP-responsive period, while their content would attenuate during the CDDP-resistant period; ΔNp73α would vary depending upon the cellular content of each tumour. Findings obtained from both ascities of fluid and solid tumours would provide insight for the development of new therapeutic strategies to overcome chemoresistance in OVCA.

5.7 Conclusions

The current thesis provides new and significant contributions regarding the cellular and molecular mechanisms of CDDP resistance in ovarian cancer cells. It is the first to demonstrate that calcium, calpain and p73α are crucial mediators in regulating OVCA cell sensitivity to CDDP. In addition, the findings of this thesis reveal that any defect in one of these mediators was enough to abrogate the whole pathway and to confer CDDP resistance. Understanding the link between these three mediators should lead to the discovery of novel strategies for the treatment of ovarian cancer, and one day, for its cure.

CHAPTER 6: REFERENCES

Abedini, M. R., E. J. Muller, et al. (2009). "Akt promotes chemoresistance in human ovarian cancer cells by modulating cisplatin-induced, p53-dependent ubiquitination of FLICE-like inhibitory protein." Oncogene.

Abedini, M. R., E. J. Muller, et al. (2008). "Cisplatin induces p53-dependent FLICE-like inhibitory protein ubiquitination in ovarian cancer cells." Cancer Res **68**(12): 4511-7.

Abedini, M. R., Q. Qiu, et al. (2004). "Possible role of FLICE-like inhibitory protein (FLIP) in chemoresistant ovarian cancer cells in vitro." Oncogene **23**(42): 6997-7004.

Abraham, R. T. (2001). "Cell cycle checkpoint signaling through the ATM and ATR kinases." Genes Dev **15**(17): 2177-96.

Agami, R., G. Blandino, et al. (1999). "Interaction of c-Abl and p73alpha and their collaboration to induce apoptosis." Nature **399**(6738): 809-13.

Ahmed, S. U., M. Bar-Peled, et al. (1997). "Cloning and subcellular location of an Arabidopsis receptor-like protein that shares common features with protein-sorting receptors of eukaryotic cells." Plant Physiol **114**(1): 325-36.

Ahn, E. Y., S. T. Lim, et al. (2004). "Calmodulin binding to the Fas death domain. Regulation by Fas activation." J Biol Chem **279**(7): 5661-6.

Amuthan, G., G. Biswas, et al. (2002). "Mitochondrial stress-induced calcium signaling, phenotypic changes and invasive behavior in human lung carcinoma A549 cells." Oncogene **21**(51): 7839-49.

Andegeko, Y., L. Moyal, et al. (2001). "Nuclear retention of ATM at sites of DNA double strand breaks." J Biol Chem **276**(41): 38224-30.

Antoniou, A., P. D. Pharoah, et al. (2003). "Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies." Am J Hum Genet **72**(5): 1117-30.

Asher, G., P. Tsvetkov, et al. (2005). "A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73." Genes Dev **19**(3): 316-21.

Asselin, E., G. B. Mills, et al. (2001). "XIAP regulates Akt activity and caspase-3 dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells." Cancer Res **61**(5): 1862-8.

Atencio, I. A., M. Ramachandra, et al. (2000). "Calpain inhibitor 1 activates p53-dependent apoptosis in tumor cell lines." Cell Growth Differ **11**(5): 247-53.
Baekelandt, M. M., R. Holm, et al. (2000). "P-glycoprotein expression is a marker for chemotherapy resistance and prognosis in advanced ovarian cancer." Anticancer Res **20**(2B): 1061-7.

Banin, S., L. Moyal, et al. (1998). "Enhanced phosphorylation of p53 by ATM in response to DNA damage." Science **281**(5383): 1674-7.

Bardella, C., D. Dettori, et al. (2007). "The therapeutic potential of hepatocyte growth factor to sensitize ovarian cancer cells to cisplatin and paclitaxel in vivo." Clin Cancer Res **13**(7): 2191-8.

Becker, K., P. Pancoska, et al. (2006). "Patterns of p73 N-terminal isoform expression and p53 status have prognostic value in gynecological cancers." Int J Oncol **29**(4): 889-902.

Beltsos, A. N. and R. R. Odem (1996). "Ovulation induction and ovarian malignancy." Semin Reprod Endocrinol **14**(4): 367-74.

Berek, J. S., K. Bertelsen, et al. (2000). "[Epithelial ovarian cancer (advanced stage): consensus conference (1998)]." Gynecol Obstet Fertil **28**(7-8): 576-83.

Bergamaschi, D., M. Gasco, et al. (2003). "p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis." Cancer Cell **3**(4): 387-402.

Bernassola, F., P. Salomoni, et al. (2004). "Ubiquitin-dependent degradation of p73 is inhibited by PML." J Exp Med **199**(11): 1545-57.

Berridge, M. J., M. D. Bootman, et al. (2003). "Calcium signalling: dynamics, homeostasis and remodelling." Nat Rev Mol Cell Biol **4**(7): 517-29.

Blair, B. G., C. A. Larson, et al. (2009). "Copper transporter 2 regulates the cellular accumulation and cytotoxicity of Cisplatin and Carboplatin." Clin Cancer Res **15**(13): 4312-21.

Brown, R., C. Clugston, et al. (1993). "Increased accumulation of p53 protein in cisplatin-resistant ovarian cell lines." Int J Cancer **55**(4): 678-84.

Buick, R. N., R. Pullano, et al. (1985). "Comparative properties of five human ovarian adenocarcinoma cell lines." Cancer Res **45**(8): 3668-76.

Canman, C. E., D. S. Lim, et al. (1998). "Activation of the ATM kinase by ionizing radiation and phosphorylation of p53." Science **281**(5383): 1677-9.

Carafoli, E. (1987). "Intracellular calcium homeostasis." Annu Rev Biochem **56**: 395-433.
Carafoli, E. (2002). "Calcium signaling: a tale for all seasons." Proc Natl Acad Sci U S A **99**(3): 1115-22.

Carragher, N. O. (2006). "Calpain inhibition: a therapeutic strategy targeting multiple disease states." Curr Pharm Des **12**(5): 615-38.

Chehab, N. H., A. Malikzay, et al. (1999). "Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage." Proc Natl Acad Sci U S A **96**(24): 13777-82.

Chen, C. L., S. M. Ip, et al. (2000). "P73 gene expression in ovarian cancer tissues and cell lines." Clin Cancer Res **6**(10): 3910-5.

Chua, B. T., K. Guo, et al. (2000). "Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases." J Biol Chem **275**(7): 5131-5.

Cramer, D. W. and W. R. Welch (1983). "Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis." J Natl Cancer Inst **71**(4): 717-21.

Crompton, M., S. Virji, et al. (1999). "The mitochondrial permeability transition pore." Biochem Soc Symp **66**: 167-79.

Cvitkovic, E. and J. L. Misset (1996). "Chemotherapy for ovarian cancer." N Engl J Med **334**(19): 1269; author reply 1270.

Dabholkar, M., J. Vionnet, et al. (1994). "Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy." J Clin Invest **94**(2): 703-8.

Danilova, N., K. M. Sakamoto, et al. (2008). "p53 family in development." Mech Dev **125**(11-12): 919-31.

Das, S., S. Nama, et al. (2005). "p73 beta-expressing recombinant adenovirus: a potential anticancer agent." Cancer Gene Ther **12**(4): 417-26.

Dhein, J., H. Walczak, et al. (1995). "Autocrine T-cell suicide mediated by APO-1/(Fas/CD95)." Nature **373**(6513): 438-41.

Di Como, C. J., C. Gaiddon, et al. (1999). "p73 function is inhibited by tumor-derived p53 mutants in mammalian cells." Mol Cell Biol **19**(2): 1438-49.

Di Sano, F., E. Ferraro, et al. (2006). "Endoplasmic reticulum stress induces apoptosis by an apoptosome-dependent but caspase 12-independent mechanism." J Biol Chem **281**(5): 2693-700.

Dijt, F. J., A. M. Fichtinger-Schepman, et al. (1988). "Formation and repair of cisplatin-induced adducts to DNA in cultured normal and repair-deficient human fibroblasts." Cancer Res **48**(21): 6058-62.

DiSaia, P. J., J. G. Sinkovics, et al. (1972). "Cell-mediated immunity to human malignant cells. A brief review and further studies with two gynecologic tumors." Am J Obstet Gynecol **114**(7): 979-89.

Dominguez, G., J. M. Garcia, et al. (2006). "DeltaTAp73 upregulation correlates with poor prognosis in human tumors: putative in vivo network involving p73 isoforms, p53, and E2F-1." J Clin Oncol **24**(5): 805-15.

Droin, N. M. and D. R. Green (2004). "Role of Bcl-2 family members in immunity and disease." Biochim Biophys Acta **1644**(2-3): 179-88.

Du, C., M. Fang, et al. (2000). "Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition." Cell **102**(1): 33-42.

Durocher, D. and S. P. Jackson (2001). "DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme?" Curr Opin Cell Biol **13**(2): 225-31.

Earnshaw, W. C., L. M. Martins, et al. (1999). "Mammalian caspases: structure, activation, substrates, and functions during apoptosis." Annu Rev Biochem **68**: 383-424.

Eastman, A. (1987). "The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes." Pharmacol Ther **34**(2): 155-66.

Easton, D. F., F. E. Matthews, et al. (1996). "Cancer mortality in relatives of women with ovarian cancer: the OPCS Study. Office of Population Censuses and Surveys." Int J Cancer **65**(3): 284-94.

el-Khateeb, M., T. G. Appleton, et al. (1999). "Reactions of cisplatin hydrolytes with methionine, cysteine, and plasma ultrafiltrate studied by a combination of HPLC and NMR techniques." J Inorg Biochem **77**(1-2): 13-21.

Elbendary, A., A. Berchuck, et al. (1994). "Transforming growth factor beta 1 can induce CIP1/WAF1 expression independent of the p53 pathway in ovarian cancer cells." Cell Growth Differ **5**(12): 1301-7.

Eltabbakh, G. H. and C. S. Awtrey (2001). "Current treatment for ovarian cancer." Expert Opin Pharmacother **2**(1): 109-24.

Enari, M., H. Sakahira, et al. (1998). "A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD." Nature **391**(6662): 43-50.

Fang, L., S. W. Lee, et al. (1999). "Comparative analysis of p73 and p53 regulation and effector functions." J Cell Biol **147**(4): 823-30.

Fathalla, M. F. (1971). "Incessant ovulation-a factor in ovarian neoplasia?" Lancet 2: 163.
Fontemaggi, G., I. Kela, et al. (2002). "Identification of direct p73 target genes combining DNA microarray and chromatin immunoprecipitation analyses." J Biol Chem **277**(45): 43359-68.

Fraser, M., T. Bai, et al. (2008). "Akt promotes cisplatin resistance in human ovarian cancer cells through inhibition of p53 phosphorylation and nuclear function." Int J Cancer **122**(3): 534-46.

Fraser, M., S. L. Chan, et al. (2006). "Regulation of p53 and suppression of apoptosis by the soluble guanylyl cyclase/cGMP pathway in human ovarian cancer cells." Oncogene **25**(15): 2203-12.

Fraser, M., B. M. Leung, et al. (2003). "p53 is a determinant of X-linked inhibitor of apoptosis protein/Akt-mediated chemoresistance in human ovarian cancer cells." Cancer Res **63**(21): 7081-8.

Fulco, M., A. Costanzo, et al. (2003). "p73 is regulated by phosphorylation at the G2/M transition." J Biol Chem **278**(49): 49196-202.

Furuya, K., T. Ozaki, et al. (2007). "Stabilization of p73 by nuclear IkappaB kinase-alpha mediates cisplatin-induced apoptosis." J Biol Chem **282**(25): 18365-78.

Gaiddon, C., M. Lokshin, et al. (2003). "Cyclin-dependent kinases phosphorylate p73 at threonine 86 in a cell cycle-dependent manner and negatively regulate p73." J Biol Chem **278**(30): 27421-31.

Gatei, M., K. Sloper, et al. (2003). "Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation." J Biol Chem **278**(17): 14806-11.

Gelebart, P., T. Kovacs, et al. (2002). "Expression of endomembrane calcium pumps in colon and gastric cancer cells. Induction of SERCA3 expression during differentiation." J Biol Chem **277**(29): 26310-20.

Gerasimenko, J. V., O. V. Gerasimenko, et al. (2002). "Menadione-induced apoptosis: roles of cytosolic Ca(2+) elevations and the mitochondrial permeability transition pore." J Cell Sci **115**(Pt 3): 485-97.

Gibb, R. K., D. D. Taylor, et al. (1997). "Apoptosis as a measure of chemosensitivity to cisplatin and taxol therapy in ovarian cancer cell lines." Gynecol Oncol **65**(1): 13-22.

Gil-Parrado, S., A. Fernandez-Montalvan, et al. (2002). "Ionomycin-activated calpain triggers apoptosis. A probable role for Bcl-2 family members." J Biol Chem **277**(30): 27217-26.

Glading, A., P. Chang, et al. (2000). "Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway." J Biol Chem **275**(4): 2390-8.

Glading, A., F. Uberall, et al. (2001). "Membrane proximal ERK signaling is required for M-calpain activation downstream of epidermal growth factor receptor signaling." J Biol Chem **276**(26): 23341-8.

Gong, J. G., A. Costanzo, et al. (1999). "The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage." Nature **399**(6738): 806-9.

Gonzalez, S., C. Prives, et al. (2003). "p73alpha regulation by Chk1 in response to DNA damage." Mol Cell Biol **23**(22): 8161-71.

Gordon, A. N., L. Asmar, et al. (2004). "Phase II study of sequential doublets: topotecan and carboplatin, followed by paclitaxel and carboplatin, in patients with newly diagnosed advanced ovarian cancer." Gynecol Oncol **94**(2): 533-9.

Gordon, A. N., M. Tonda, et al. (2004). "Long-term survival advantage for women treated with pegylated liposomal doxorubicin compared with topotecan in a phase 3 randomized study of recurrent and refractory epithelial ovarian cancer." Gynecol Oncol **95**(1): 1-8.

Goto, S., K. Yoshida, et al. (1995). "Augmentation of transport for cisplatin-glutathione adduct in cisplatin-resistant cancer cells." Cancer Res **55**(19): 4297-301.

Goudelock, D. M., K. Jiang, et al. (2003). "Regulatory interactions between the checkpoint kinase Chk1 and the proteins of the DNA-dependent protein kinase complex." J Biol Chem **278**(32): 29940-7.

Gourdier, I., L. Crabbe, et al. (2004). "Oxaliplatin-induced mitochondrial apoptotic response of colon carcinoma cells does not require nuclear DNA." Oncogene **23**(45): 7449-57.

Grob, T. J., U. Novak, et al. (2001). "Human delta Np73 regulates a dominant negative feedback loop for TAp73 and p53." Cell Death Differ **8**(12): 1213-23.

Guan, M. and Y. Chen (2005). "Aberrant expression of DeltaNp73 in benign and malignant tumours of the prostate: correlation with Gleason score." J Clin Pathol **58**(11): 1175-9.

Gurney, A. L., S. A. Marsters, et al. (1999). "Identification of a new member of the tumor necrosis factor family and its receptor, a human ortholog of mouse GITR." Curr Biol **9**(4): 215-8.

Harms, K. L. and X. Chen (2006). "The functional domains in p53 family proteins exhibit both common and distinct properties." Cell Death Differ **13**(6): 890-7.

Harriman, J. F., X. L. Liu, et al. (2002). "Endoplasmic reticulum Ca(2+) signaling and calpains mediate renal cell death." Cell Death Differ **9**(7): 734-41.

Hirao, A., Y. Y. Kong, et al. (2000). "DNA damage-induced activation of p53 by the checkpoint kinase Chk2." Science **287**(5459): 1824-7.

Holcakova, J., P. Ceskova, et al. (2008). "The cell type-specific effect of TAp73 isoforms on the cell cycle and apoptosis." Cell Mol Biol Lett **13**(3): 404-20.

Holschneider, C. H. and J. S. Berek (2000). "Ovarian cancer: epidemiology, biology, and prognostic factors." Semin Surg Oncol **19**(1): 3-10.

Holzer, A. K., G. H. Manorek, et al. (2006). "Contribution of the major copper influx transporter CTR1 to the cellular accumulation of cisplatin, carboplatin, and oxaliplatin." Mol Pharmacol **70**(4): 1390-4.

Honda, R., H. Tanaka, et al. (1997). "Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53." FEBS Lett **420**(1): 25-7.

Horiuchi, A., C. Wang, et al. (2007). "BRCA1 Expression is an Important Biomarker for Chemosensitivity: Suppression of BRCA1 Increases the Apoptosis via Up-regulation of p53 and p21 During Cisplatin Treatment in Ovarian Cancer Cells." Biomark Insights **1**: 49-59.

Hu, J. F., G. A. Ulaner, et al. (2000). "Allelic expression of the putative tumor suppressor gene p73 in human fetal tissues and tumor specimens." Biochim Biophys Acta **1491**(1-3): 49-56.

Huang, J. B., A. L. Kindzelskii, et al. (2004). "Identification of channels promoting calcium spikes and waves in HT1080 tumor cells: their apparent roles in cell motility and invasion." Cancer Res **64**(7): 2482-9.

Irmler, M., M. Thome, et al. (1997). "Inhibition of death receptor signals by cellular FLIP." Nature **388**(6638): 190-5.

Irwin, M. S. (2004). "Family feud in chemosensitvity: p73 and mutant p53." Cell Cycle **3**(3): 319-23.

Irwin, M. S., K. Kondo, et al. (2003). "Chemosensitivity linked to p73 function." Cancer Cell **3**(4): 403-10.

Ishimoto, O., C. Kawahara, et al. (2002). "Possible oncogenic potential of DeltaNp73: a newly identified isoform of human p73." Cancer Res **62**(3): 636-41.

Jackson, S. P. (1997). "DNA-dependent protein kinase." Int J Biochem Cell Biol **29**(7): 935-8.

Jayaraman, T. and A. R. Marks (1997). "T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis." Mol Cell Biol **17**(6): 3005-12.

Jones, E. V., M. J. Dickman, et al. (2007). "Regulation of p73-mediated apoptosis by c-Jun N-terminal kinase." Biochem J **405**(3): 617-23.

Jordan, P. and M. Carmo-Fonseca (2000). "Molecular mechanisms involved in cisplatin cytotoxicity." Cell Mol Life Sci **57**(8-9): 1229-35.

Kaghad, M., H. Bonnet, et al. (1997). "Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers." Cell **90**(4): 809-19.

Kaku, T., S. Ogawa, et al. (2003). "Histological classification of ovarian cancer." Med Electron Microsc **36**(1): 9-17.

Kalayda, G. V., C. H. Wagner, et al. (2008). "Altered localisation of the copper efflux transporters ATP7A and ATP7B associated with cisplatin resistance in human ovarian carcinoma cells." BMC Cancer **8**: 175.

Kamazawa, S., J. Kigawa, et al. (2002). "Multidrug resistance gene-1 is a useful predictor of Paclitaxel-based chemotherapy for patients with ovarian cancer." Gynecol Oncol **86**(2): 171-6.

Kanamori, Y., J. Kigawa, et al. (1998). "A newly developed adenovirus-mediated transfer of a wild-type p53 gene increases sensitivity to cis-diamminedichloroplatinum (II) in p53-deleted ovarian cancer cells." Eur J Cancer **34**(11): 1802-6.

Kartasheva, N. N., A. Contente, et al. (2002). "p53 induces the expression of its antagonist p73 Delta N, establishing an autoregulatory feedback loop." Oncogene **21**(31): 4715-27.

Katoh, I., K. I. Aisaki, et al. (2000). "p51A (TAp63gamma), a p53 homolog, accumulates in response to DNA damage for cell regulation." Oncogene **19**(27): 3126-30.

Kaufmann, S. H. and W. C. Earnshaw (2000). "Induction of apoptosis by cancer chemotherapy." Exp Cell Res **256**(1): 42-9.

Kaufmann, S. H. and G. J. Gores (2000). "Apoptosis in cancer: cause and cure." Bioessays **22**(11): 1007-17.

Kelland, L. R. (1993). "New platinum antitumor complexes." Crit Rev Oncol Hematol **15**(3): 191-219.

Kerr, J. F., A. H. Wyllie, et al. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**(4): 239-57.

Kigawa, J., S. Sato, et al. (2002). "Effect of p53 gene transfer and cisplatin in a peritonitis carcinomatosa model with p53-deficient ovarian cancer cells." Gynecol Oncol **84**(2): 210-5.

Kim, S. T., D. S. Lim, et al. (1999). "Substrate specificities and identification of putative substrates of ATM kinase family members." J Biol Chem **274**(53): 37538-43.

Kobayashi, S., K. Yamashita, et al. (2002). "Calpain-mediated X-linked inhibitor of apoptosis degradation in neutrophil apoptosis and its impairment in chronic neutrophilic leukemia." J Biol Chem **277**(37): 33968-77.

Komatsu, M., T. Sumizawa, et al. (2000). "Copper-transporting P-type adenosine triphosphatase (ATP7B) is associated with cisplatin resistance." Cancer Res **60**(5): 1312-6.

Korosec, B., D. Glavac, et al. (2006). "Alterations in the ATP2A2 gene in correlation with colon and lung cancer." Cancer Genet Cytogenet **171**(2): 105-11.

Kulesz-Martin, M., J. Lagowski, et al. (2005). "Melanocyte and keratinocyte carcinogenesis: p53 family protein activities and intersecting mRNA expression profiles." J Investig Dermatol Symp Proc **10**(2): 142-52.

Kuo, M. T., H. H. Chen, et al. (2007). "The roles of copper transporters in cisplatin resistance." Cancer Metastasis Rev **26**(1): 71-83.

Lane, D. P. and L. V. Crawford (1979). "T antigen is bound to a host protein in SV40-transformed cells." Nature **278**(5701): 261-3.

Lane, R. D., D. M. Allan, et al. (1992). "A comparison of the intracellular distribution of mu-calpain, m-calpain, and calpastatin in proliferating human A431 cells." Exp Cell Res **203**(1): 5-16.

Lee, S., E. J. Choi, et al. (2005). "Activation of PI3K/Akt pathway by PTEN reduction and PIK3CA mRNA amplification contributes to cisplatin resistance in an ovarian cancer cell line." Gynecol Oncol **97**(1): 26-34.

Lehtovirta, P. (2000). "[Surgical treatment of ovarian cancer should be centralized]." Duodecim **116**(10): 1033-5.

Leitao, M. M., R. A. Soslow, et al. (2004). "Mutation and expression of the TP53 gene in early stage epithelial ovarian carcinoma." Gynecol Oncol **93**(2): 301-6.

Letai, A., M. C. Bassik, et al. (2002). "Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics." Cancer Cell **2**(3): 183-92.

Leung, E. L., M. Fraser, et al. (2008). "Cisplatin alters nitric oxide synthase levels in human ovarian cancer cells: involvement in p53 regulation and cisplatin resistance." Br J Cancer **98**(11): 1803-9.

Li, L. Y., X. Luo, et al. (2001). "Endonuclease G is an apoptotic DNase when released from mitochondria." Nature **412**(6842): 95-9.

Li, Q., J. J. Yu, et al. (2000). "Association between the level of ERCC-1 expression and the repair of cisplatin-induced DNA damage in human ovarian cancer cells." Anticancer Res **20**(2A): 645-52.

Liang, P. F., P. H. Zhang, et al. (2004). "[Experimental study on the induction of endothelial apoptosis by burn serum and subeschar tissue fluid]." Zhonghua Shao Shang Za Zhi **20**(5): 275-7.

Liao, J., A. Schneider, et al. (2006). "Extracellular calcium as a candidate mediator of prostate cancer skeletal metastasis." Cancer Res **66**(18): 9065-73.

Lin, X., T. Okuda, et al. (2002). "The copper transporter CTR1 regulates cisplatin uptake in Saccharomyces cerevisiae." Mol Pharmacol **62**(5): 1154-9.

Lin, Y. L., S. Sengupta, et al. (2009). "p63 and p73 transcriptionally regulate genes involved in DNA repair." PLoS Genet **5**(10): e1000680.

Lindahl, T., P. Karran, et al. (1997). "DNA excision repair pathways." Curr Opin Genet Dev **7**(2): 158-69.

Liu, G., S. Nozell, et al. (2004). "DeltaNp73beta is active in transactivation and growth suppression." Mol Cell Biol **24**(2): 487-501.

Liu, L., D. Xing, et al. (2008). "Calpain-mediated pathway dominates cisplatin-induced apoptosis in human lung adenocarcinoma cells as determined by real-time single cell analysis." Int J Cancer **122**(10): 2210-22.

Liu, S. S., K. Y. Chan, et al. (2006). "Expression of deltaNp73 and TAp73alpha independently associated with radiosensitivities and prognoses in cervical squamous cell carcinoma." Clin Cancer Res **12**(13): 3922-7.

Liu, X., H. Zou, et al. (1997). "DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis." Cell **89**(2): 175-84.

Lockshin, R. A. and C. M. Williams (1965). "Programmed Cell Death--I. Cytology of Degeneration in the Intersegmental Muscles of the Pernyi Silkmoth." J Insect Physiol **11**: 123-33.

Lorenzo, H. K., S. A. Susin, et al. (1999). "Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death." Cell Death Differ **6**(6): 516-24.

Lowndes, N. F. and J. R. Murguia (2000). "Sensing and responding to DNA damage." Curr Opin Genet Dev **10**(1): 17-25.

Maisse, C., E. Munarriz, et al. (2004). "DNA damage induces the rapid and selective degradation of the DeltaNp73 isoform, allowing apoptosis to occur." Cell Death Differ **11**(6): 685-7.

Mamenta, E. L., E. E. Poma, et al. (1994). "Enhanced replicative bypass of platinum-DNA adducts in cisplatin-resistant human ovarian carcinoma cell lines." Cancer Res **54**(13): 3500-5.

Mandic, A., J. Hansson, et al. (2003). "Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling." J Biol Chem **278**(11): 9100-6.

Mandic, A., K. Viktorsson, et al. (2002). "Calpain-mediated Bid cleavage and calpain-independent Bak modulation: two separate pathways in cisplatin-induced apoptosis." Mol Cell Biol **22**(9): 3003-13.

Mansouri, A., Q. Zhang, et al. (2003). "Cisplatin resistance in an ovarian carcinoma is associated with a defect in programmed cell death control through XIAP regulation." Oncol Res **13**(6-10): 399-404.

Marabese, M., S. Marchini, et al. (2008). "Expression levels of p53 and p73 isoforms in stage I and stage III ovarian cancer." Eur J Cancer **44**(1): 131-41.

Marchini, S., M. Marabese, et al. (2008). "DeltaNp63 expression is associated with poor survival in ovarian cancer." Ann Oncol **19**(3): 501-7.

Marrazzo, E., S. Marchini, et al. (2009). "The expression of the DeltaNp73beta isoform of p73 leads to tetraploidy." Eur J Cancer **45**(3): 443-53.

Matsuoka, S., M. Huang, et al. (1998). "Linkage of ATM to cell cycle regulation by the Chk2 protein kinase." Science **282**(5395): 1893-7.

McCollum, A. T., P. Nasr, et al. (2002). "Calpain activates caspase-3 during UV-induced neuronal death but only calpain is necessary for death." J Neurochem **82**(5): 1208-20.

Melino, G., F. Bernassola, et al. (2004). "p73 Induces apoptosis via PUMA transactivation and Bax mitochondrial translocation." J Biol Chem **279**(9): 8076-83.

Melino, G., X. Lu, et al. (2003). "Functional regulation of p73 and p63: development and cancer." Trends Biochem Sci **28**(12): 663-70.

Miyamoto, S., A. L. Howes, et al. (2005). "Ca2+ dysregulation induces mitochondrial depolarization and apoptosis: role of Na+/Ca2+ exchanger and AKT." J Biol Chem **280**(46): 38505-12.

Mlynarczuk-Bialy, I., H. Roeckmann, et al. (2006). "Combined effect of proteasome and calpain inhibition on cisplatin-resistant human melanoma cells." Cancer Res **66**(15): 7598-605.

Modan, B., P. Hartge, et al. (2001). "Parity, oral contraceptives, and the risk of ovarian cancer among carriers and noncarriers of a BRCA1 or BRCA2 mutation." N Engl J Med **345**(4): 235-40.

Moggs, J. G., D. E. Szymkowski, et al. (1997). "Differential human nucleotide excision repair of paired and mispaired cisplatin-DNA adducts." Nucleic Acids Res **25**(3): 480-91.
Moll, U. M. (2003). "The Role of p63 and p73 in tumor formation and progression: coming of age toward clinical usefulness. Commentary re: F. Koga et al., Impaired p63 expression associates with poor prognosis and uroplakin III expression in invasive urothelial carcinoma of the bladder. Clin. Cancer Res., 9: 5501-5507, 2003, and P. Puig et al., p73 Expression in human normal and tumor tissues: loss of p73alpha expression is associated with tumor progression in bladder Cancer. Clin. Cancer Res., 9: 5642-5651, 2003." Clin Cancer Res **9**(15): 5437-41.

Moll, U. M. and N. Slade (2004). "p63 and p73: roles in development and tumor formation." Mol Cancer Res **2**(7): 371-86.

Moncoq, K., C. A. Trieber, et al. (2007). "The molecular basis for cyclopiazonic acid inhibition of the sarcoplasmic reticulum calcium pump." J Biol Chem **282**(13): 9748-57.

Monteith, G. R., D. McAndrew, et al. (2007). "Calcium and cancer: targeting Ca2+ transport." Nat Rev Cancer **7**(7): 519-30.

Mujoo, K., L. Zhang, et al. (2000). "Emergence of cisplatin-resistant cells from the OVCAR-3 ovarian carcinoma cell line with p53 mutations, altered tumorigenicity, and increased apoptotic sensitivity to p53 gene replacement." Int J Gynecol Cancer **10**(2): 105-114.

Muller, M., T. Schilling, et al. (2005). "TAp73/Delta Np73 influences apoptotic response, chemosensitivity and prognosis in hepatocellular carcinoma." Cell Death Differ **12**(12): 1564-77.

Muller, M., E. S. Schleithoff, et al. (2006). "One, two, three--p53, p63, p73 and chemosensitivity." Drug Resist Updat **9**(6): 288-306.

Munarriz, E., D. Bano, et al. (2005). "Calpain cleavage regulates the protein stability of p73." Biochem Biophys Res Commun **333**(3): 954-60.

Murray-Zmijewski, F., D. P. Lane, et al. (2006). "p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress." Cell Death Differ **13**(6): 962-72.

Musat, M., V. V. Vax, et al. (2004). "Cell cycle dysregulation in pituitary oncogenesis." Front Horm Res **32**: 34-62.

Muscolini, M., R. Cianfrocca, et al. (2008). "Trichostatin A up-regulates p73 and induces Bax-dependent apoptosis in cisplatin-resistant ovarian cancer cells." Mol Cancer Ther **7**(6): 1410-9.

Nakagawa, T., M. Takahashi, et al. (2002). "Autoinhibitory regulation of p73 by Delta Np73 to modulate cell survival and death through a p73-specific target element within the Delta Np73 promoter." Mol Cell Biol **22**(8): 2575-85.

Nakagawa, T. and J. Yuan (2000). "Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis." J Cell Biol **150**(4): 887-94.

Nakayama, K., A. Kanzaki, et al. (2004). "Prognostic value of the Cu-transporting ATPase in ovarian carcinoma patients receiving cisplatin-based chemotherapy." Clin Cancer Res **10**(8): 2804-11.

Ng, C. P., H. C. Lee, et al. (2004). "Differential mode of regulation of the checkpoint kinases CHK1 and CHK2 by their regulatory domains." J Biol Chem **279**(10): 8808-19.

Ng, S. W., G. K. Yiu, et al. (2000). "Analysis of p73 in human borderline and invasive ovarian tumor." Oncogene **19**(15): 1885-90.

Oguchi, H., F. Kikkawa, et al. (1994). "Glutathione related enzymes in cis-diamminedichloroplatinum (II)-sensitive and-resistant human ovarian carcinoma cells." Anticancer Res **14**(1A): 193-200.

Okada, Y., N. Fukasawa, et al. (2002). "Atlanto-axial subluxation (Grisel's syndrome) associated with mumps." Pediatr Int **44**(2): 192-4.

Ono, Y., N. Nonomura, et al. (2001). "Loss of p73 induction in a cisplatin-resistant bladder cancer cell line." Mol Urol **5**(1): 25-30.

Ozaki, T., M. Hosoda, et al. (2005). "Functional implication of p73 protein stability in neuronal cell survival and death." Cancer Lett **228**(1-2): 29-35.

Ozaki, T. and A. Nakagawara (2005). "p73, a sophisticated p53 family member in the cancer world." Cancer Sci **96**(11): 729-37.

Ozaki, T., R. Okoshi, et al. (2009). "Acetylation status of E2F-1 has an important role in the regulation of E2F-1-mediated transactivation of tumor suppressor p73." Biochem Biophys Res Commun **386**(1): 207-11.

Perrin, B. J. and A. Huttenlocher (2002). "Calpain." Int J Biochem Cell Biol **34**(7): 722-5.
Petitjean, A., C. Ruptier, et al. (2008). "Properties of the six isoforms of p63: p53-like regulation in response to genotoxic stress and cross talk with DeltaNp73." Carcinogenesis **29**(2): 273-81.

Pietsch, E. C., S. M. Sykes, et al. (2008). "The p53 family and programmed cell death." Oncogene **27**(50): 6507-21.

Pinton, P., D. Ferrari, et al. (2001). "The Ca2+ concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action." Embo J **20**(11): 2690-701.

Plooy, A. C., M. van Dijk, et al. (1985). "Formation and repair of DNA interstrand cross-links in relation to cytotoxicity and unscheduled DNA synthesis induced in control and mutant human cells treated with cis-diamminedichloroplatinum(II)." Cancer Res **45**(9): 4178-84.

Poli Neto, O. B., F. J. Candido Dos Reis, et al. (2006). "p63 expression in epithelial ovarian tumors." Int J Gynecol Cancer **16**(1): 152-5.

Porter, A. G., P. Ng, et al. (1997). "Death substrates come alive." Bioessays **19**(6): 501-7.
Poulain, L., H. Lincet, et al. (1998). "Acquisition of chemoresistance in a human ovarian carcinoma cell is linked to a defect in cell cycle control." Int J Cancer **78**(4): 454-63.

Putney, J. W., Jr., L. M. Broad, et al. (2001). "Mechanisms of capacitative calcium entry." J Cell Sci **114**(Pt 12): 2223-9.

Ramadan, S., A. Terrinoni, et al. (2005). "p73 induces apoptosis by different mechanisms." Biochem Biophys Res Commun **331**(3): 713-7.

Reed, E. (1998). "Platinum-DNA adduct, nucleotide excision repair and platinum based anti-cancer chemotherapy." Cancer Treat Rev **24**(5): 331-44.

Ren, J., R. Datta, et al. (2002). "p73beta is regulated by protein kinase Cdelta catalytic fragment generated in the apoptotic response to DNA damage." J Biol Chem **277**(37): 33758-65.

Righetti, S. C., P. Perego, et al. (2008). "Cooperation between p53 and p73 in cisplatin-induced apoptosis in ovarian carcinoma cells." Cancer Lett **263**(1): 140-4.

Rock, M. T., A. R. Dix, et al. (2000). "Beta1 integrin-mediated T cell adhesion and cell spreading are regulated by calpain." Exp Cell Res **261**(1): 260-70.

Rosenberg, B., L. Vancamp, et al. (1965). "Inhibition of Cell Division in Escherichia Coli by Electrolysis Products from a Platinum Electrode." Nature **205**: 698-9.

Rossi, M., V. De Laurenzi, et al. (2005). "The ubiquitin-protein ligase Itch regulates p73 stability." Embo J **24**(4): 836-48.

Sabatino, M. A., S. Previdi, et al. (2007). "In vivo evaluation of the role of DNp73alpha protein in regulating the p53-dependent apoptotic pathway after treatment with cytotoxic drugs." Int J Cancer **120**(3): 506-13.

Samimi, G., N. M. Varki, et al. (2003). "Increase in expression of the copper transporter ATP7A during platinum drug-based treatment is associated with poor survival in ovarian cancer patients." Clin Cancer Res **9**(16 Pt 1): 5853-9.

Sanchez-Prieto, R., V. J. Sanchez-Arevalo, et al. (2002). "Regulation of p73 by c-Abl through the p38 MAP kinase pathway." Oncogene **21**(6): 974-9.

Sang, M., K. Ando, et al. (2009). "Plk3 inhibits pro-apoptotic activity of p73 through physical interaction and phosphorylation." Genes Cells **14**(7): 775-88.

Saraste, A. and K. Pulkki (2000). "Morphologic and biochemical hallmarks of apoptosis." Cardiovasc Res **45**(3): 528-37.

Sasaki, H., Y. Sheng, et al. (2000). "Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells." Cancer Res **60**(20): 5659-66.

Sayan, A. E., A. Paradisi, et al. (2005). "New antibodies recognizing p73: comparison with commercial antibodies." Biochem Biophys Res Commun **330**(1): 186-93.

Sayan, A. E., B. S. Sayan, et al. (2008). "P73 and caspase-cleaved p73 fragments localize to mitochondria and augment TRAIL-induced apoptosis." Oncogene **27**(31): 4363-72.

Sedarous, M., E. Keramaris, et al. (2003). "Calpains mediate p53 activation and neuronal death evoked by DNA damage." J Biol Chem **278**(28): 26031-8.

Selvakumaran, M., D. A. Pisarcik, et al. (2003). "Enhanced cisplatin cytotoxicity by disturbing the nucleotide excision repair pathway in ovarian cancer cell lines." Cancer Res **63**(6): 1311-6.

Shan, Y., W. Sheng, et al. (2009). "[Adenovirus mediated IL-24 gene expression inhibits growth of human glioma cell in vitro]." Sheng Wu Gong Cheng Xue Bao **25**(2): 279-86.

Shaw, T. J., M. K. Senterman, et al. (2004). "Characterization of intraperitoneal, orthotopic, and metastatic xenograft models of human ovarian cancer." Mol Ther **10**(6): 1032-42.

Shi, Y. (2001). "A structural view of mitochondria-mediated apoptosis." Nat Struct Biol **8**(5): 394-401.

Shimada, M., J. Kigawa, et al. (2000). "Mechanism of the combination effect of wild-type TP53 gene transfection and cisplatin treatment for ovarian cancer xenografts." Eur J Cancer **36**(14): 1869-75.

Shiraha, H., A. Glading, et al. (1999). "IP-10 inhibits epidermal growth factor-induced motility by decreasing epidermal growth factor receptor-mediated calpain activity." J Cell Biol **146**(1): 243-54.

Siddik, Z. H. (2003). "Cisplatin: mode of cytotoxic action and molecular basis of resistance." Oncogene **22**(47): 7265-79.

Soussi, T. (2003). "[p53 mutations and resistance to chemotherapy: a stab in the back for p73?]." Bull Cancer **90**(5): 383-6.

Splettstoesser, F., A. M. Florea, et al. (2007). "IP(3) receptor antagonist, 2-APB, attenuates cisplatin induced Ca2+-influx in HeLa-S3 cells and prevents activation of calpain and induction of apoptosis." Br J Pharmacol **151**(8): 1176-86.

Steren, A., B. U. Sevin, et al. (1993). "Taxol sensitizes human ovarian cancer cells to radiation." Gynecol Oncol **48**(2): 252-8.

Stiewe, T. (2007). "The p53 family in differentiation and tumorigenesis." Nat Rev Cancer **7**(3): 165-8.

Stiewe, T., C. C. Theseling, et al. (2002). "Transactivation-deficient Delta TA-p73 inhibits p53 by direct competition for DNA binding: implications for tumorigenesis." J Biol Chem **277**(16): 14177-85.

Strano, S., E. Munarriz, et al. (2000). "Physical and functional interaction between p53 mutants and different isoforms of p73." J Biol Chem **275**(38): 29503-12.

Suzuki, Y., Y. Imai, et al. (2001). "A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death." Mol Cell **8**(3): 613-21.

Tanaka, Y., M. Kameoka, et al. (2004). "Regulation of HSF1-responsive gene expression by N-terminal truncated form of p73alpha." Biochem Biophys Res Commun **317**(3): 865-72.

Tanaka, Y., K. Ota, et al. (2006). "Up-regulation of NFkappaB-responsive gene expression by DeltaNp73alpha in p53 null cells." Exp Cell Res **312**(8): 1254-64.

Tartaglia, L. A., M. Rothe, et al. (1993). "Tumor necrosis factor's cytotoxic activity is signaled by the p55 TNF receptor." Cell **73**(2): 213-6.

Tewari, M., L. T. Quan, et al. (1995). "Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase." Cell **81**(5): 801-9.

Thebault, S., L. Lemonnier, et al. (2005). "Novel role of cold/menthol-sensitive transient receptor potential melastatine family member 8 (TRPM8) in the activation of store-operated channels in LNCaP human prostate cancer epithelial cells." J Biol Chem **280**(47): 39423-35.

Thomas, L. R., A. Henson, et al. (2004). "Direct binding of Fas-associated death domain (FADD) to the tumor necrosis factor-related apoptosis-inducing ligand receptor DR5 is regulated by the death effector domain of FADD." J Biol Chem **279**(31): 32780-5.

Torigoe, T., H. Izumi, et al. (2005). "Cisplatin resistance and transcription factors." Curr Med Chem Anticancer Agents **5**(1): 15-27.

Tortolero-Luna, G. and M. F. Mitchell (1995). "The epidemiology of ovarian cancer." J Cell Biochem Suppl **23**: 200-7.

Toyota, H., N. Yanase, et al. (2003). "Calpain-induced Bax-cleavage product is a more potent inducer of apoptotic cell death than wild-type Bax." Cancer Lett **189**(2): 221-30.
Trope, C., T. Paulsen, et al. (2000). "[Controversies in surgical treatment of advanced ovarian cancer]." Tidsskr Nor Laegeforen **120**(7): 824-30.

Tsavaler, L., M. H. Shapero, et al. (2001). "Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins." Cancer Res **61**(9): 3760-9.

Tullio, R. D., M. Passalacqua, et al. (1999). "Changes in intracellular localization of calpastatin during calpain activation." Biochem J **343 Pt 2**: 467-72.

Tuve, S., T. Racek, et al. (2006). "Adenovirus-mediated TA-p73beta gene transfer increases chemosensitivity of human malignant melanomas." Apoptosis **11**(2): 235-43.

Uramoto, H., K. Sugio, et al. (2004). "Expression of deltaNp73 predicts poor prognosis in lung cancer." Clin Cancer Res **10**(20): 6905-11.

Vikhanskaya, F., M. D'Incalci, et al. (2000). "p73 competes with p53 and attenuates its response in a human ovarian cancer cell line." Nucleic Acids Res **28**(2): 513-9.

Vikhanskaya, F., S. Marchini, et al. (2001). "P73a overexpression is associated with resistance to treatment with DNA-damaging agents in a human ovarian cancer cell line." Cancer Res **61**(3): 935-8.

Vo, C. and M. E. Carney (2007). "Ovarian cancer hormonal and environmental risk effect." Obstet Gynecol Clin North Am **34**(4): 687-700, viii.

Wang, J., T. Wiltshire, et al. (2004). "ATM-dependent CHK2 activation induced by anticancer agent, irofulven." J Biol Chem **279**(38): 39584-92.

Wang, J. Y. (1998). "Cellular responses to DNA damage." Curr Opin Cell Biol **10**(2): 240-7.

Wang, L., J. Mao, et al. (2007). "Nano-cerium-element-doped titanium dioxide induces apoptosis of Bel 7402 human hepatoma cells in the presence of visible light." World J Gastroenterol **13**(29): 4011-4.

Wang, Q. W., H. Liu, et al. (2008). "[Proteasome inhibitor bortezomib sensitizes Hep-2 human laryngeal squamous cell carcinoma cells to ionizing radiation in vitro and in vivo]." Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi **43**(6): 456-9.

Wei, M. C., T. Lindsten, et al. (2000). "tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c." Genes Dev **14**(16): 2060-71.

Whittemore, A. S. (1993). "Personal characteristics relating to risk of invasive epithelial ovarian cancer in older women in the United States." Cancer **71**(2 Suppl): 558-65.

Wolf, C. R., I. P. Hayward, et al. (1987). "Cellular heterogeneity and drug resistance in two ovarian adenocarcinoma cell lines derived from a single patient." Int J Cancer **39**(6): 695-702.

Wood, D. E. and E. W. Newcomb (2000). "Cleavage of Bax enhances its cell death function." Exp Cell Res **256**(2): 375-82.

Wood, D. E., A. Thomas, et al. (1998). "Bax cleavage is mediated by calpain during drug-induced apoptosis." Oncogene **17**(9): 1069-78.

Wright, J. A., K. S. Keegan, et al. (1998). "Protein kinase mutants of human ATR increase sensitivity to UV and ionizing radiation and abrogate cell cycle checkpoint control." Proc Natl Acad Sci U S A **95**(13): 7445-50.

Wu, G., S. Nomoto, et al. (2003). "DeltaNp63alpha and TAp63alpha regulate transcription of genes with distinct biological functions in cancer and development." Cancer Res **63**(10): 2351-7.

Yan, X., M. Fraser, et al. (2006). "Over-expression of PTEN sensitizes human ovarian cancer cells to cisplatin-induced apoptosis in a p53-dependent manner." Gynecol Oncol **102**(2): 348-55.

Yang, A., M. Kaghad, et al. (1998). "p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities." Mol Cell **2**(3): 305-16.

Yang, D., S. S. van Boom, et al. (1995). "Structure and isomerization of an intrastrand cisplatin-cross-linked octamer DNA duplex by NMR analysis." Biochemistry **34**(39): 12912-20.

Yang, H., L. He, et al. (2006). "Aurora-A induces cell survival and chemoresistance by activation of Akt through a p53-dependent manner in ovarian cancer cells." Int J Cancer **119**(10): 2304-12.

Yang, J., Z. P. Xu, et al. (2004). "ATM and ATR: sensing DNA damage." World J Gastroenterol **10**(2): 155-60.

Yang, J. C. and G. A. Cortopassi (1998). "Induction of the mitochondrial permeability transition causes release of the apoptogenic factor cytochrome c." Free Radic Biol Med **24**(4): 624-31.

Yang, X., M. Fraser, et al. (2008). "Regulation of apoptosis-inducing factor-mediated, cisplatin-induced apoptosis by Akt." Br J Cancer **98**(4): 803-8.

Yang, X., M. Fraser, et al. (2006). "Akt-mediated cisplatin resistance in ovarian cancer: modulation of p53 action on caspase-dependent mitochondrial death pathway." Cancer Res **66**(6): 3126-36.

Yang, Y. P., H. S. Kuo, et al. (2005). "The p53-dependent apoptotic pathway of breast cancer cells (BC-M1) induced by the bis-type bioreductive compound aziridinylnaphthoquinone." Breast Cancer Res **7**(1): R19-27.

Yoshida, I., A. Monji, et al. (2006). "Depletion of intracellular Ca2+ store itself may be a major factor in thapsigargin-induced ER stress and apoptosis in PC12 cells." Neurochem Int **48**(8): 696-702.

Yoshida, K., T. Ozaki, et al. (2008). "ATM-dependent nuclear accumulation of IKK-alpha plays an important role in the regulation of p73-mediated apoptosis in response to cisplatin." Oncogene **27**(8): 1183-8.

Zaika, A., M. Irwin, et al. (2001). "Oncogenes induce and activate endogenous p73 protein." J Biol Chem **276**(14): 11310-6.

Zaika, A. I., N. Slade, et al. (2002). "DeltaNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors." J Exp Med **196**(6): 765-80.

Zamble, D. B. and S. J. Lippard (1995). "Cisplatin and DNA repair in cancer chemotherapy." Trends Biochem Sci **20**(10): 435-9.

Zhang, B., J. S. Pan, et al. (2006). "Effects of chemotherapy and/or radiotherapy on survivin expression in ovarian cancer." Methods Find Exp Clin Pharmacol **28**(9): 619-25.

Zhao, H. and H. Piwnica-Worms (2001). "ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1." Mol Cell Biol **21**(13): 4129-39.

Zou, H., Y. Li, et al. (1999). "An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9." J Biol Chem **274**(17): 11549-56.

CHAPTRR 7: APPENDICES

7.1 CDDP decreased TAp73β content in chemosensitive cells (OV2008) but not in its resistant counterpart (C13)*

Previously, we showed that CDDP decreased TAp73α and ΔNp73α contents in OV2008 but not in C13*. We thought it would be interesting to examine the effect of CDDP on another isoform of the p73 group; TAp73β. We treated OV2008 and C13* with CDDP (10 μM; 24 h) and TAp7β content was assessed by Western Blot. The effect of CDDP on TAp7β content was similar to the p73α isoforms in which it decreased its content in OV2008 but not C13*. However, we are not sure if the CDDP-induced, TAp7β down-regulation in OV2008 is mediated by calpain as for p73α isoforms (Figure 4.8). Although, calpain has been shown to cleave all p73 isoforms including TAp7β (Munarriz, Bano et al. 2005), similar approaches of calpain inhibition should be used to address such concern.

Figure 7.1: The effect of CDDP on TAp73β contents in OVCA cells.

Chemosensitive (OV2008) cells and its resistant counterparts (C13*) were treated with CDDP (10 μM; 24 h) or DMSO (control). TAp73β and GAPDH contents were measured by Western blot. N = 3

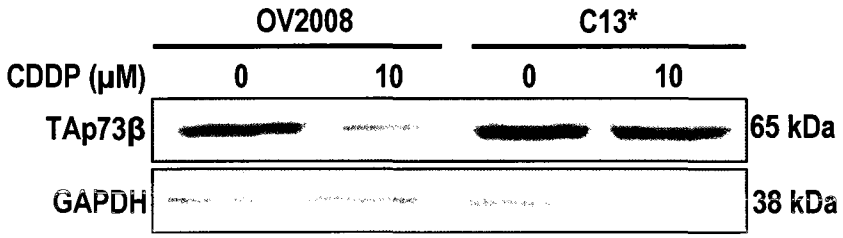


Figure 7.1

7.2 Taxol decreased TAp73α and ΔNp73α protein contents in both chemosensitive and chemoresistant OVCA cells

We demonstrated that CDDP decreased both TAp73α and ΔNp73α contents in chemosensitive cells (O2008) but not in its resistant counterpart (C13*) (Chapter 4; Figure 4.1A). Next, we tested if this effect is specific to CDDP or can be mediated by other chemotherapeutic agents such as Taxol. Taxol is a mitotic inhibitor used in combination with CDDP for treating ovarian cancer (mentioned in Chapter 1). In this context, both OV2008 and C13* were treated with different concentrations of CDDP (0, 2.5, 5 and 10 μM) and Taxol (0, 5, 10 and 20 μM). Cells were harvested 24 hours post-treatment and TAp73α, ΔNp73α and GAPDH contents were detected by Western blot. As expected, CDDP induced TAp73α and ΔNp73α down-regulation in OV2008 but not C13* in a concentration-dependent manner (Figure 4.1). Interesting, Taxol induced such response in both cell lines which were not consistent with a previously published paper where they showed that Taxol and CDDP had no effect on p73 content in OVCA cells (2780) (Vikhanskaya, D'Incalci *et al.* 2000) (discussed in Chapter 5).

Although our findings suggested that both CDDP and Taxol have similar effect on TAp73α and ΔNp73α content, we are not sure if both isoforms are also regulated by the same mechanisms (i.e. Ca^{2+}/calpain pathway) in drugs-induced apoptosis. Therefore, further investigations are required to assess the mechanism by which Taxol regulates TAp73α and ΔNp73α content in Taxol-induced apoptosis in OVCA cells.

Figure 7.2: The effect of CDDP and Taxol on TAp73α and ΔNp73α content in OVCA cells.

Chemosensitive (OV2008) and Chemoresistant (C13*) cells were treated with different concentrations of CDDP (0, 2.5, 5 and 10 μM) and Taxol (0, 5, 10 and 20 μM). Cells were harvested 24 hours post-treatment and TAp73α, ΔNp73α and GAPDH contents were detected by Western blot. N = 3

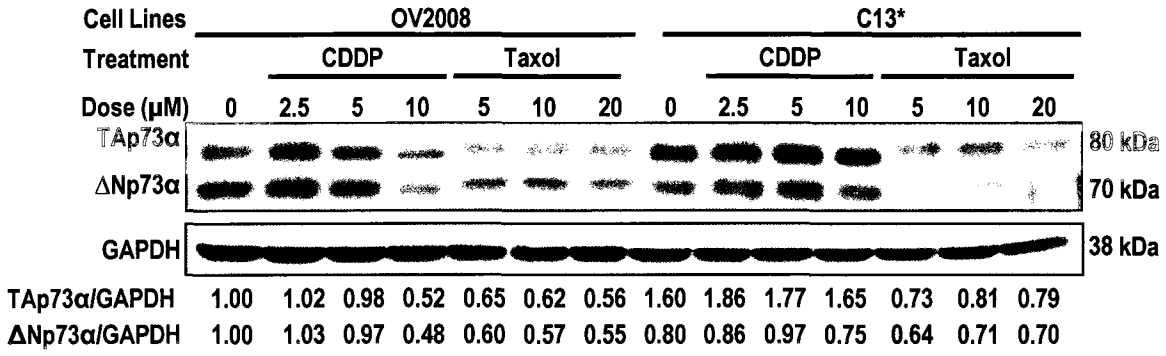

Cell Lines
OV2008
C13*
Treatment
CDDP
Taxol
CDDP
Taxol
Dose (μM)
0 2.5 5 10 5 10 20 0 2.5 5 10 5 10 20
TAp73α
ΔNp73α
80 kDa
70 kDa
GAPDH
38 kDa
TAp73α/GAPDH 1.00 1.02 0.98 0.52 0.65 0.62 0.56 1.60 1.86 1.77 1.65 0.73 0.81 0.79
ΔNp73α/GAPDH 1.00 1.03 0.97 0.48 0.60 0.57 0.55 0.80 0.86 0.97 0.75 0.64 0.71 0.70

Figure 7.2

7.3 The increase in TAp73α and ΔNp73α content over time could be mediated by phosphorylation in chemosensitive OVCA cells.

An interesting phenomenon was observed in which TAp73α and ΔNp73α content was increasing over the duration of the culture period in chemosensitive (OV2008) but not in chemoresistant (C13*) cells (Figure 4.4). It has been shown that p73 expression is modulated during the cell cycle and its protein accumulate at the S phase cells (Fulco, Costanzo *et al.* 2003). In addition, the same investigators with others (Gaiddon, Lokshin *et al.* 2003) demonstrated that p73 is phosphorylated by cyclin-dependent kinases (CDK) at theronine ($T^{86}PEH$) in cell cycle-dependent manner in different cell lines: HCT116 (colon carcinoma), U2OS (osteosarcoma), MCF7 (breast cancer), and HeLa (cervix carcinoma). In this context, we speculated that the increase in both isoforms content in these cells might be due to post-translational modification such as phosphorylation in relation to cell growth.

In order to test this speculation; we first confirmed that the bands representing both TAp73α and ΔNp73α without any treatment are phosphorylated. Such step was achieved by incubating the cell-lysate obtained from OV2008 cells cultured over 24 hours with three different types of phosphatases (New-England; USA) at 30° C for 1 hour. These are: Calf Intestinal Alkaline phosphatase (CIAP; general phosphatase), Protein Phosphatase 1 (PP1; Serine/Threonine phosphorylation) and Protein Tyrosine Phosphatase (LAR; Tyrosine phosphorylation). In figure 4.7A, a band shift was observed when both CIAP and PP1 were used while there was no effect for the LAR. These results suggested that both TAp73α and ΔNp73α are phosphorylated and specifically at the Serine/Threonine sites.

To further provide evidence that TAp73α and ΔNp73α are phosphorylated overtime in OV2008 cells, TAp73α and ΔNp73α were immunoprecipitated at different time point (0, 6, 12 and 24 h) as described in chapter 3. Then, a specific threonine adjacent to proline antibody was used to detect both isoforms phosphorylation. As shown in Figure 4.7B, TAp73α and ΔNp73α isoforms were successfully pull-down where their phosphorylation was detected.

Once we showed that TAp73α and ΔNp73α were phosphorylated at Threonine sites, we next determined if such phosphorylation might be mediated by the kinase; $p34^{cdc2}$. OV2008 cells were transfected with $p34^{cdc2}$ siRNA (0-100 nM; Santa Cruz) into and then treated with CDDP (10 μM) to reduce the content of $p34^{cdc2}$ and thus inhibit p73α phosphorylation. As shown in Figure 4.8, $p34^{cdc2}$ siRNA significantly reduces its content in both CDDP-treated and non-treated groups compared to the control siRNA. CDDP-induced apoptosis was enhanced when $p34^{cdc2}$ content was reduced. Both TAp73α and ΔNp73α content was down-regulated, an observation consistent with $p34^{cdc2}$ decreased. P53 content was used as a positive control in which $p34^{cdc2}$ down-regulation by siRNA had no effect on its content.

In contrast, TAp73α and ΔNp73α content was not increasing over the duration of the culture period. We speculated that the lack of $p34^{cdc2}$-dependent, p73α phosphorylation in chemoresistant (C13*) OVCA cells might be due to defect in cell cycle control as previously published (Poulain, Lincet *et al.* 1998). They found that the acquisition of chemoresistance after CDDP exposure in ovarian cancer cells affects cell-cycle checkpoints including CDK inhibitors. Further works are required to test this

speculation including, the assessement of different phases of the cell cycle and their regulators (i. e. CDK) in these cells and compare it to the chemosensitive (OV2008) cells.

Taken together, our findings partially support the hypothesis that the increase in TAp73α and ΔNp73α content without any treatment might be mediated by $p34^{cdc2}$. However, additional experiments are required to fully support the above hypothesis including, the use of specific antibody against the theronine ($T^{86}PEH$) site on TAp73α and ΔNp73α and site-directed mutagenesis to confirm it. In addition, examining the cell cycle phases of OV2008 cells by flow cytometry and relating these phase to TAp73α and ΔNp73α phosphorylation will be very helpful in providing further support for the above hypothesis.

In summary, although we showed that p73α is involved in CDDP-induced apoptosis, these findings suggested that it might also be regulated by CDKs in cell-cycle progression and different post-translational modifications of its protein might determine its function in both cell-cycle and apoptosis.

Figure 7.3: TAp73α and ΔNp73α phosphorylation and the effect of phosphatases on their content in chemosensitive cells.

A) Three different types of phosphatases; Calf Intestinal Alkaline phosphatase (CIAP), Protein Phosphatase 1 (PP1) and Protein Tyrosine Phosphatase (LAR) were incubated (30° C, 1 h) with cell-lysate obtained from OV2008 cells cultured over 24 hours. Finally, TAp73α, ΔNp73α and venculin content were detected by Western Blot.

B) OV2008 cells were cultured over twenty four hours duration and harvested at different time point (0, 6, 12 and 24 h). TAp73α and ΔNp73α were then immunoprecipitated as described in Chapter 3 and their phosphorylation was detected using specific threonine/proline antibody. Total and phosphoryated TAp73α and ΔNp73α were detected by Western blot. N = 3.

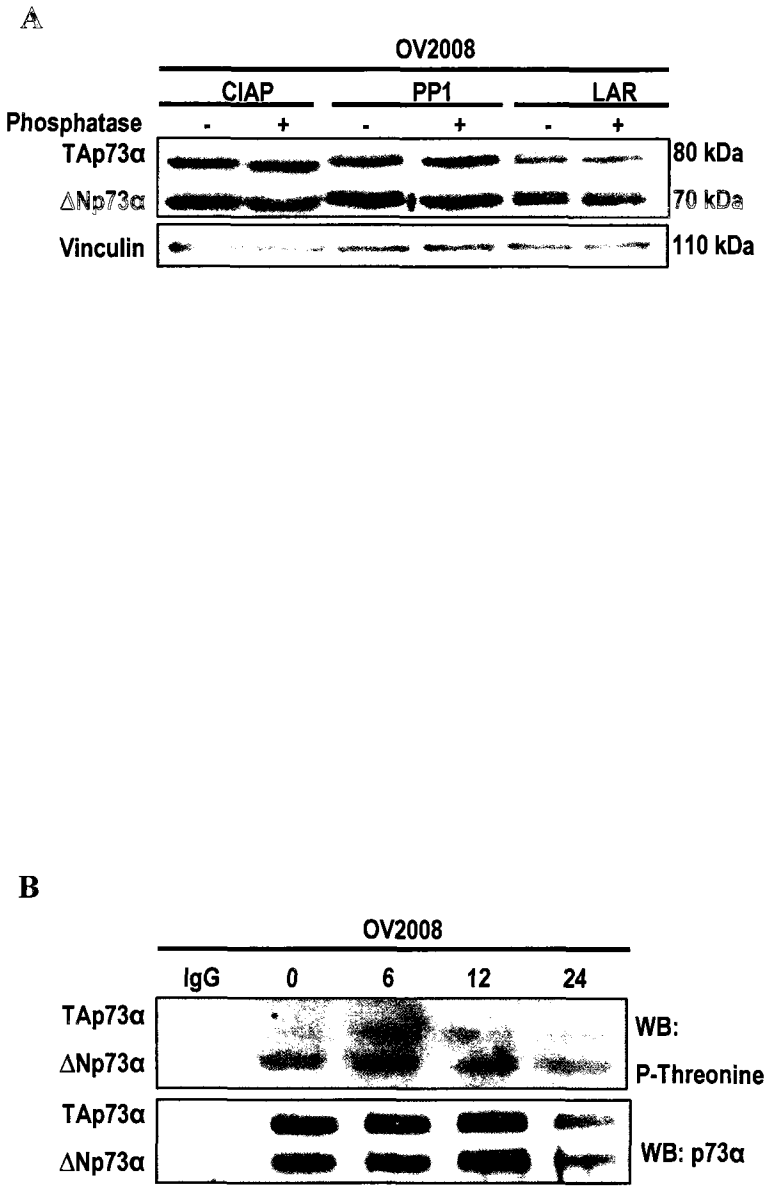

A
OV2008
CIAP
PP1
LAR
Phosphatase
-
+
-
+
-
+
TAp73α
ΔNp73α
Vinculin
80 kDa
70 kDa
110 kDa
B
OV2008
IgG
0
6
12
24
TAp73α
ΔNp73α
WB:
P-Threonine
TAp73α
ΔNp73α
WB: p73α
IP: p73α

Figure 7.3

Figure 7.4: The effect of $p34^{cdc2}$ siRNA on TAp73α and ΔNp73α content and CDDP-induced apoptosis in chemosensitive cells.

Chemosensitive OVCA cells (OV2008) were transfected with $p34^{cdc2}$ siRNA (0-100 nM) or a control siRNA and treated 24 hours thereafter with CDDP (10 μM) or DMSO for 24 hours. $P34^{cdc2}$, p53, TAp73α, ΔNp73α and GAPDH contents and apoptosis were measured by Western blot and Hoechst staining respectively. Data are represented as the mean ± SEM of three independent experiments. Two way-ANOVA indicates a significant CDDP effect ($P < 0.001$), $p34^{cdc2}$ siRNA effect ($P < 0.05$) and significant CDDP- $p34^{cdc2}$ siRNA interaction ($P < 0.05$). Bonferroni post hoc test shows significant differences between control siRNA and $p34^{cdc2}$ siRNA ($P < 0.05$) in CDDP-treated groups.

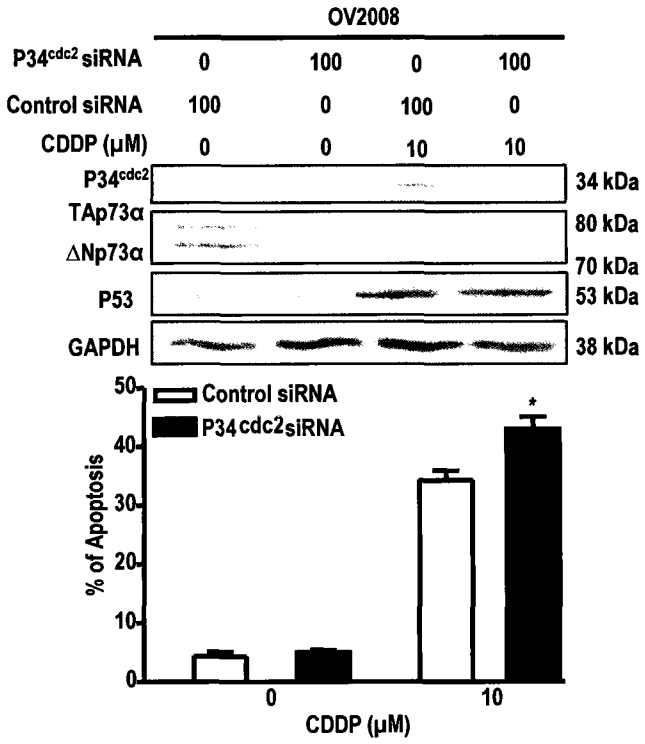

OV2008
P34cdc2 siRNA
0
100
0
100
Control siRNA
100
0
100
0
CDDP (μM)
0
0
10
10
P34cdc2
34 kDa
TAp73α
80 kDa
ΔNp73α
70 kDa
P53
53 kDa
GAPDH
38 kDa
Control siRNA
P34cdc2siRNA
% of Apoptosis
50
40
30
20
10
0
*
0
10
CDDP (μM)

Figure 7.4

CHAPTER 8: CURRICULUM VITAE

Shadia Al Bahlani
Ph.D. Candidate, University of Ottawa
Ottawa Hospital Research Institute
Ottawa, Ontario, Canada

EDUCATION

01/2005–present	Ph.D. Candidate, Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada
09/2001–09/2002	M.Sc. in Molecular Pathology (Pass with Distinction), University of Leicester, Leicester, United Kindom
09/1994-06/1999	B.Sc. in Medical Laboratory Science (Histopathology), Sultan Qaboos University, Al-Khoud, Sultanate of Oman

WORK EXPERIENCE

01/2005–present	Graduate student, Reproductive Biology Unit, Department of Obstetrics & Gynaecology and Cellular & Molecular Medicine, University of Ottawa, Chronic Disease Program, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada
10/2002–present	Lecturer, Department of Pathology, College of Medicine and Health Sciences, Sultan Qaboos University, Oman
10/1999–09/2001	Laboratory demonstrator, Department of Pathology, College of Medicine and Health Sciences, Sultan Qaboos University, Oman
09/1999-10/1999	Demonstrator in Human & Clinical Anatomy Department, College of medicine and Health Sciences.

AWARD AND DISTINCTIONS

2005-2009	Graduate Scholarship Award (PhD.), Sultan Qaboos University, Sultanate of Oman.
2001-2002	Distinction Award, MSc. in Molecular Pathology, University of Leicester, Leicester, United Kingdom.
2001-2002	Graduate Scholarship Award (MSc.), Sultan Qaboos University, Sultanate of Oman.

CONFERENCE ATTENDANCE

12/1998	Biomedical Sciences: Towards the Millennium, MLS Symposium. Institute of Health Sciences,. Oman
01/2000	Advanced Medicine Symposium and 1st Genetics Symposium, Sultan Qaboos University, Oman
03/2000	1st International Congress in Tropical & infectious disease" Al.Bustan Palace, Oman
06/2000	Leukemia in Children" Grand Hayatt, Oman
02/2001	14th International Child Health Conference, Grand Hayatt, Oman
12/2002	3th GCC Conference of Faculties of Medicine on Medical Education, Sultan Qaboos University, Oman
10/2003	Gene Cloning and DNA Analysis" workshop, Arabian Gulf University, Kingdom of Bahrain
5/2005	23 Annual Ottawa Reproductive Biology Workshop, Ottawa, ON, Canada
5/2006	24 Annual Ottawa Reproductive Biology Workshop, Ottawa, ON, Canada
5/2006	3rd National Conference on Ovarian Cancer Research, Vancouver, British Columbia, Canada
5/2007	25 Annual Ottawa Reproductive Biology Workshop, Ottawa, ON, Canada

5/2008	26 Annual Ottawa Reproductive Biology Workshop, Ottawa, ON, Canada
5/2009	27 Annual Ottawa Reproductive Biology Workshop, Ottawa, ON, Canada
8/2009	4th p63/p73 Workshop, Toronto, ON, Canada

LABORATORY SKILLS

- **Anatomical Techniques:** Cadavers dissection.
- **Histological Techniques:** Tissue processing, sectioning and staining.
- **Molecular and Cellular Techniques:** Cell culture, Western blotting, Reverse transcriptase polymerase chain reaction (RT-PCR), Immunocytochemistry, Immunoprecipitation and Calcium Imaging.

PLATFORM PRESENTATIONS

1. **Al Bahlani S.,** and Tsang B.K. P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain-dependent mechanism. 9th Annual OHRI Research Day, Ottawa, ON, Canada, 2009

2. **Al Bahlani S.,** and Tsang B.K. P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain-dependent mechanism. 27th Annual Ottawa Reproductive Biology Workshop, Ottawa, ON, Canada, 2009

PUBLISHED ABSTRACTS

1. **Al Bahlani S.,** and Tsang B.K. CDDP-induced, p73α down-regulation is calpain-dependent in human ovarian cancer. 4th p63/p73 Workshop, Toronto, ON, Canada, 2009

2. **Al Bahlani S.,** Fraser M., and Tsang B.K. The role of p73 in CDDP-induced apoptosis in human ovarian cancer. 26th Annual Ottawa Reproductive Biology Workshop, Ottawa, ON, Canada, 2008

3. **Al Bahlani S.,** Fraser M., Tricia Lin, and Tsang B.K. The role of p73 in CDDP-induced apoptosis in human ovarian cancer. Making Connections: A Canadian Research Conference, Toronto, ON, Canada, 2007

4. **Al Bahlani S.,** Fraser M., and Tsang B.K. The role of p73 in CDDP-induced apoptosis in human ovarian cancer. 25th Annual Ottawa Reproductive Biology Workshop, Ottawa, ON, Canada, 2007. Awarded as the second place best poster

5. **Al Bahlani S.,** Fraser M., and Tsang B.K. The role of p73 in CDDP-induced apoptosis in human ovarian cancer. 3rd National Conference on Ovarian Cancer Research, Vancouver, British Columbia, Canada

6. **Al Bahlani S.,** Fraser M., and Tsang B.K. The role of p73 in CDDP-induced apoptosis in human ovarian cancer. 24th Annual Ottawa Reproductive Biology Workshop, Ottawa, ON, Canada, 2006

7. **Al Bahlani S.,** Fraser M., and Tsang B.K. The role of p73 in CDDP-induced apoptosis in human ovarian cancer. 5th Annual OHRI Research Day, Ottawa, ON, Canada, 2005

8. **Al Bahlani S.,** Fraser M., and Tsang B.K. The role of p73 in CDDP-induced apoptosis in human ovarian cancer. 23th Annual Ottawa Reproductive Biology Workshop, Ottawa, ON, Canada, 2005

9. **Al Bahlani S.,** Al Rawahi S., and Al Hutti R. The role of sulphomucin in the progression of Intestinal Metaplasia, Sultan Qaboos University Research Day, Oman, 2003

10. **Al Bahlani S.,** and Shaw J. Analysis of Genetic alteration in the epithelial and stromal cells of breast cancer. The Pathological Society meeting of Great Britain and Ireland, United Kingdom, 2003

11. Rao K., Kaplan E., and **Al Bahlani S.** A very rare case of Amyopathic Lateral Sclerosis with Systemic Lupus Erythematosus, College of Medicine and Health Sciences Research Day, Oman, 2000

12. **Al Bahlani S.,** and Al Rawahi S. Cytological Specimen Reception, Health and Safety Awareness Week, Oman, 2001. Awarded as the 2nd best poster presented

13. Al Rawahi S., **Al Bahlani S.,** and Kaplan E. A comparison study of Helicobacter Pylori detection methods and the assessment of the associated pathological condition, College of Medicine and Health Sciences Research Day, Oman, 2001. Awarded as the first best poster presented

MANUSCRIPTS SUBMITTED

Shadia M Al Bahlani, Adrian Y.C. Wong, Michael Fraser, Berna S. Sayan, Richard Bergeron, Gerry Melino and Benjamin K. Tsang (2010). P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain-dependent mechanism.

ASSIGNED MENTORSHIP

06/2007-08/2007 Tricia Lin, Undergraduate summer student, Reproductive Biology Unit, Chronic Disease Program, Ottawa Health Research Institute.